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Published in:
Default journal

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 268, No. 9, Issue of March 25, pp. 6323-6331, 1993
Printed in U.S.A.

Cell-permeable Non-hydrolyzable cAMP Derivatives as Tools for Analysis of Signaling Pathways Controlling Gene Regulation in *Dictyostelium**

(Received for publication, October 5, 1992)

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A novel class of cAMP derivatives were tested for binding to surface cAMP receptors (CAR), protein kinase A (PKA), and cAMP-phosphodiesterase (PDE) and for induction of three classes of cAMP regulated genes in *Dictyostelium discoideum*. These derivatives carry sulfur substitutions for either the axial (Sp) or equatorial (Rp) exocyclic oxygen atoms, while further modifications were introduced to provide specificity for binding to either CAR or PKA, and/or to increase lipophilicity and render the derivatives membrane-permeable. All derivatives bind weakly to PDE and are almost not degraded during incubation with *Dictyostelium* cells. One cAMP derivative, 6-thioethylpurineriboside 3′,5′-monophosphorothioate, Sp-isomer (Sp-6SEtcPuMPS), fulfills the criteria for selective activation of PKA *in vivo*. The compound enters *Dictyostelium* cells and reaches an intracellular concentration of 1 μM, sufficient to activate PKA, at an extracellular concentration of 30 μM, which is insufficient to activate CAR. Expression of cAMP-regulated prespore and prestalk genes and the aggregative PDE gene are effectively induced by CAR agonists and very poorly by PKA agonists. Even Sp-6SEtcPuMPS is ineffective to induce gene expression. These data not only indicate that surface cAMP receptors are the first targets for cAMP-induced gene expression, but argue against direct induction of expression of these genes by cAMP-induced PKA activation.

During *Dictyostelium discoideum* development extracellular cAMP regulates expression of several classes of genes. In preaggregative cells, nanomolar cAMP pulses enhance expression of aggregative genes, coding for *e.g.* surface cAMP receptors, adhesive contact sites A, a guanine nucleotide regulatory protein (G-protein)[1] α-subunit (Gα2), and cAMP-phosphodiesterase (PDE) (Darmon *et al.*, 1975; Gerisch *et al.*, 1975; Lacombe *et al.*, 1986; Noegel *et al.*, 1986; Klein *et al.*, 1988; Kumagai *et al.*, 1989). Expression of PDE can additionally be induced by a constant cAMP stimulus (Yeh *et al.*, 1978). After aggregation, persistent stimulation with micromolar cAMP concentrations induces the expression of prespore genes and of a subclass of prestalk genes (Mehdy *et al.*, 1983; Schaap and Van Driel, 1985; Oyama and Blumberg, 1986).

Dictyostelium cells contain several putative targets for the effects of cAMP. At present four genes coding for highly homologous surface cAMP receptors (CARs) have been cloned (Klein *et al.*, 1988; Saxe *et al.*, 1991). These receptors show seven putative transmembrane domains, characteristic of receptors interacting with G-proteins. Two intracellular cAMP-binding proteins have been cloned; the regulatory (R) subunit of a cAMP-dependent protein kinase (PKA) (Mutzel *et al.*, 1987) and a hitherto unknown cAMP-binding protein (CABP1) (Grant and Tsang, 1990). The *Dictyostelium* PKA R-subunit is very similar to its mammalian counterpart, both in amino acid sequence and cyclic nucleotide specificity. The cyclic nucleotide specificity of purified CABP1 protein is virtually identical to the PKA R-subunit (Van Ments-Cohen *et al.*, 1989), but the deduced amino acid sequence of its cloned gene is completely dissimilar from the R-subunit sequence, and does not contain the consensus sequence for cAMP binding (Grant and Tsang, 1990).

Comparison of nucleotide specificity of different cAMP-induced responses and the specificity of *Dictyostelium* cAMP-binding proteins, using 20 systematically modified cAMP derivatives, has shown that cAMP-induced accumulation of second messengers and chemotaxis are mediated by CARs (Konijn, 1973; De Wit *et al.*, 1982; Van Haastert and Kien, 1983; Theibert *et al.*, 1986). Using a limited number of cAMP derivatives, it was demonstrated that nucleotide specificity of induction of prespore and prestalk gene expression is similar to that of CAR and dissimilar from PKA and CABP1 (Schaap and Van Driel, 1985; Oyama and Blumberg, 1986; Haribabu and Dottin, 1986; Gomer *et al.*, 1986). It was furthermore shown that mutants defective in activation of adenylyl cyclase

* This research was supported by Grant 900-546-077 and by the C. and C. Huygens Fund from the Netherlands Organization for Scientific Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: G-protein, guanine nucleotide regulatory protein; PDE, cAMP-phosphodiesterase; CAR, cAMP receptor; PKA, protein kinase A; HPLC, high performance liquid chromatography; ePDE, extracellular cAMP phosphodiesterase.

show virtually normal induction of expression of cAMP-regulated genes (Schaap *et al.*, 1986; Bozzaro *et al.*, 1987; Mann *et al.*, 1988). These results indicate that the cAMP signal for gene induction is detected by surface cAMP receptors, and that intracellular cAMP, and therefore activation of PKA, are not involved in gene regulation.

In contrast to these conclusions, recent molecular genetic experiments strongly suggest requirement of PKA activation for gene regulation. Inactivation of PKA by overexpression of native R-subunit or R-subunit lacking the cAMP-binding sites blocks development and inhibits expression of the cAMP-induced prestalk and prespore genes CP2 and D19 (Simon *et al.*, 1989; Firtel and Chapman, 1990; Harwood *et al.*, 1992a). In these cells, the cAMP pulse-induced genes are normally expressed, and the PDE gene is overexpressed. In transformants, lacking the PKA catalytic subunit, cAMP cannot induce prespore gene expression (Mann and Firtel, 1991).[2] These data suggest that the catalytic activity of PKA is essential for positive regulation of prestalk and prespore gene expression. Further indications for involvement of PKA were derived from observations that 8-bromo-cAMP, which is a good PKA and poor CAR agonist, can induce spore differentiation (Kay, 1989; Richardson *et al.*, 1991) and expression of the aggregative contact site A gene (Ma and Siu, 1990).

Pharmacological analysis of gene regulation by cAMP derivatives requires several hours of cell exposure to cAMP (derivative), during which the agonist is degraded by extracellular PDE. cAMP degradation products as adenosine are furthermore known to exert both positive and negative effects on expression of cAMP-regulated genes (Weijer and Durston, 1985; Schaap and Wang, 1986; Spek *et al.*, 1988). Since not all cAMP derivatives are equally sensitive to degradation (Van Haastert *et al.*, 1983), and the effects of their degradation products are not known, this could result in unpredictable interference with gene expression. In general, the efficacy of poor CAR agonists will be overestimated, since at the high concentrations where these compounds are active, their degradation is more or less saturated. In order to overcome these difficulties, and to produce unequivocal evidence for or against involvement of CAR or PKA in gene regulation, we have used a novel set of cAMP derivatives. In these derivatives an axial (Sp) or equatorial (Rp) exocyclic oxygen is replaced by sulfur, which renders the cyclic phosphate ring resistant to degradation by PDE (Van Haastert *et al.*, 1983; Eckstein, 1985; Braumann *et al.*, 1986). The derivatives carry systematic modifications resulting in loss of hydrogen bonding at specific positions of the molecule, or in alteration of the *syn/anti* equilibrium. Some derivatives have additionally been modified to reduce polarity and enhance membrane permeability and are shown to enter the cell.

We here compare the affinities of these derivatives for CAR and PKA to their efficacy as agonists for induction of three classes of cAMP-regulated genes. Our results yield definite evidence for involvement of surface cAMP receptors in gene regulation and argue against involvement of PKA.

EXPERIMENTAL PROCEDURES

Materials—Rp-cAMPS, Sp-8ClcAMPS, Sp-8BrcAMPS, and Sp-5,6DClcBIMPS were obtained from Biolog Life Science Institute (Bremen, Federal Republic of Germany (F. R. G.)). Sp-6ClcPUMPS, Sp-6SEtcPUMPS and Sp-cBIMPS were synthesized according to Dostmann *et al.* (1987), Sp-5,6DBrcBIMPS, Sp-2′HcAMPS and Rp-2′HcAMPS according to Genieser *et al.* (1988), and Sp-5′NHcAMPS and Rp-5′NHcAMPS as described by Jastorff and Krebs (1972). All compounds were checked by HPLC to be free of cAMP and the corresponding diastereoisomers. The absolute configuration at phosphorus was assigned by ^{31}P NMR (Rp downfield of Sp) and the elution pattern (Rp before Sp) from a Lichrosorb RP18 HPLC column. The lipophilicity was determined as log K_w values (Braumann and Jastorff, 1985). Sp-cAMPS was obtained from Boehringer (F. R. G.), G418, dithiothreitol (dithiothreitol), *o*-nitrophenyl β-D-galactopyranoside, *Ophiophagus hanna* snake venom, and Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) were from Sigma. [2,8-^{3}H]cAMP (1.92 TBq/mmol) and [γ-^{32}P]ATP (185 TBq/mmol) were obtained from Amersham (United Kingdom).

Cell Culture and Incubation Conditions—*D. discoideum* strain NC4 was grown in association with *Escherichia coli* 281 on glucose/peptone agar and strain AX3 in HL5 liquid medium (Ashworth and Watts, 1970). To obtain aggregation competent cells, exponentially growing cells were washed with 10 mM sodium/potassium phosphate buffer, pH 6.5 (PB) to remove bacteria or nutrient medium, distributed on PB agar at 3×10^6 cells/cm^2 and incubated for 16 h at 6 °C. To obtain slugs, cells were incubated on PB agar for 16–18 h at 22 °C.

D. discoideum strain AX2 transformed with vector pA6PTlac1 containing a gene fusion of the D19 prespore promoter and the *E. coli lacZ* gene (D19-*lacZ*) (Dingermann *et al.*, 1989) and strain AX3 transformed with vectors containing, respectively, a gene fusion of the *lacZ* gene and the aggregative PDE promoter (PDE$_{ag}$-*lacZ*) (Faure *et al.*, 1990),[3] or the CP2 prestalk promoter (CP2-*lacZ*) (Pears and Williams, 1987; Datta and Firtel, 1988)[4] were grown in HL5 medium supplemented with 10 μg/ml G418. All three cell lines show correct developmental and cAMP-induced β-galactosidase accumulation, synchronous with the synthesis of the authentic mRNAs (data not shown).

For gene induction experiments PDE$_{ag}$-*lacZ* cells were harvested from growth medium, resuspended in 10 mM PB to 10^6 cells/ml, and incubated as 100-μl aliquots in microtiter plate wells. CP2-*lacZ* cells were first starved for 4 h on PB agar at 3×10^6 cells/cm^2, subsequently resuspended to 3×10^6 cells/ml, and incubated as 100-μl aliquots. D19-*lacZ* cells were starved for 16 h at 6 °C, resuspended to 3×10^6 cells/ml, and incubated as 30-μl aliquots. Microtiter plates were covered with moist filter paper and were shaken for 0.5 s with 4-s intervals on an Eppendorff mixer (model 5432) for 6 h at 20 °C. cAMP derivatives were added once at the onset of the incubation period.

Isolation of cAMP-dependent Protein Kinase Holoenzyme—The PKA holoenzyme of *D. discoideum* was isolated from slug cells as described by Schoen *et al.* (1984) with some alterations. Migrating slugs were harvested, washed once in PB, and dissociated into single cells by a 15-min treatment with cellulase (5 mg/ml) and 2 mM EDTA in PB at 22 °C (Wang *et al.*, 1988). All subsequent steps were performed at 4 °C. Cells were washed twice in lysis buffer (10 mM Tris-HCl, pH 7.6, 7 mM $MgCl_2$, 2 mM EDTA, 250 mM sucrose, and 10 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 150 μg/ml benzamidine, 50 μg/ml leupeptine, and 20 μg/ml antipain) and lysed by filtration through a Nuclepore filter (pore size 3 μM). Unbroken cells and membranes were removed by centrifugation for 10 min at 10,000 × *g*. The supernatant was centrifuged again in a Beckman Airfuge at 100,000 × *g* for 5 min. The supernatant was applied immediately on a Sephacryl 300 (Pharmacia) gel filtration column and eluted with lysis buffer, as described by Schoen *et al.* (1984). The procedure for isolation of the regulatory subunit of PKA has been described earlier (Tsang and Tasaka, 1986). The cAMP binding peak which eluted from the DE52 (Whatman) anion-exchange column at 200 mM NaCl was used to test the binding specificity of cAMP derivatives to the regulatory subunit.

Protein Kinase A Binding and Activity Assays—The affinity of cAMP derivatives for the PKA R-subunit was measured as described before (De Wit *et al.*, 1982). Activation of partially purified PKA holoenzyme was measured using the heptapeptide Kemptide as synthetic substrate (De Gunzburg *et al.*, 1984). The assays were performed at 30 °C in a final volume of 50 μl containing 50 mM MOPS buffer, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EGTA, 20 mM NaF, 0.2 mM Kemptide, 0.1 mM [γ-^{32}P]ATP (50 counts·min^{-1}·pmol), 30 μl of PKA holoenzyme preparation, and various concentrations of cAMP derivatives. After 10 min the reaction was terminated by the addition of 25 μl of glacial acetic acid. The phospho-Kemptide was separated from unreacted [γ-^{32}P]ATP and inorganic phosphate by means of P81 phosphocellulose paper (Whatman).

Binding of Derivatives to CAR and PDE—The affinity of cAMP

[2] S. O. Mann and R. A. Firtel, manuscript submitted.

[3] A. L. Hall and R. N. Kessin, manuscript in preparation.

[4] C. Gaskins and R. A. Firtel, unpublished results.

derivatives for cell surface receptors was measured as described by Van Haastert and De Wit (1984). In short, 8×10^6 aggregation competent cells were incubated with 10 mM dithiothreitol, 3 nM [^{3}H]cAMP and different concentrations of cAMP or derivatives in a total volume of 50 μl of PB. After incubation for 60 s at 4 °C, cells were separated from unbound ligand by centrifugation through silicone oil. Nonspecific binding was measured by adding 0.1 mM cAMP to the incubation mixture.

An extracellular cAMP-phosphodiesterase (ePDE) preparation was obtained by incubating 200 ml of 2×10^7 cells/ml of *D. discoideum* NC4 for 20 h with a 1 μmol·liter^{-1}·min^{-1} influx of cAMP. Cells and debris were removed by centrifugation for 5 min at 200 × *g*. The ePDE was precipitated with 44% (w/v) $(NH_4)_2SO_4$ and centrifuged for 30 min at 30,000 × *g*. The pellet was rinsed once with 44% $(NH_4)_2SO_4$, dissolved in 5 ml of PB, containing 0.1% bovine serum albumin, and concentrated by Amicon filtration to 0.5 ml. The preparation contained 73 IU of PDE activity/ml. To measure the IC_{50} (concentration inducing half-maximal inhibition of [^{3}H]cAMP degradation) of phosphorothioates for ePDE, 5 μl of 1:80,000 diluted ePDE preparation was incubated for 30 min at 22 °C with 1 μM [^{3}H]cAMP and various concentrations of phosphorothioates in a total volume of 20 μl. The reaction was terminated by boiling. The mixture was incubated for 30 min at 37 °C with 25 μl of 1 mg/ml snake venom, containing 5′-nucleotidase, shaken for 2 min with a Dowex AG 1X2 (Serva, F. R. G.) slurry, and centrifuged for 2 min at 9,000 × *g*. The radioactivity of the supernatant was determined. Assay blanks, obtained by adding boiled ePDE to the reaction mixture, were subtracted.

Degradation of Derivatives by Dictyostelium Cells—Aggregation competent cells were resuspended to 3×10^6 cells/ml in PB and incubated for 4 h with 10 μM of cAMP or phosphorothioate derivative. At the indicated time intervals, 20-μl aliquots were removed, boiled for 2 min, and assayed by means of HPLC using a Lichrospher-100 RP18 column (Merck, F. R. G.) and isocratic elution with 15% methanol in 50 mM tetraethylammoniumformate, pH 6.5.

Uptake of cAMP Derivatives—Aggregation competent cells were resuspended to 3×10^8 cells/ml in PB. 90 μl of cell suspension was shaken for 0 or 45 min with 30 μM of derivative. The cells were washed four times with 1.5 ml of PB by centrifugation in an Eppendorf centrifuge at 300 × *g*, resuspended in 50 μl of PB, and lysed with 50 μl of perchloric acid (3.5%, v/v). After neutralization with 25 μl of 50% saturated $KHCO_3$, the amount of cAMP derivative was measured by isotope dilution assay, using the regulatory subunit of the PKA as the cAMP-binding protein (Van Haastert, 1984). The derivative concentrations were calculated, using a standard curve of inhibition of [^{3}H]cAMP binding by the various derivatives.

β-Galactosidase Assay—Cells in microtiter plate wells were lysed by freeze-thawing. 25 μl of 2.5 × Z buffer (25 mM KCl, 7.5 mM $MgCl_2$, 1% (v/v) mercaptoethanol in 250 mM sodium phosphate, pH 7.0) was added to 100 μl cell lysate, and the enzyme reaction was started by addition of 25 μl of 10 mg/ml *o*-nitrophenyl β-D-galactopyranoside. When sufficient yellow reaction product had formed (about 0.5 OD_{415} units) the reaction was terminated with 100 μl of 2.5 M Na_2CO_3. The OD_{415} of the wells was measured using a Bio-Rad microtiterplate reader.

RESULTS

Rationale for the Selection of cAMP Derivatives—From the recently synthesized adenosine 3′5′-monophosphorothioate derivatives (Jastorff and Krebs, 1972; Dostmann, 1987; Genieser *et al.*, 1988), we chose a subset of compounds which were aimed to particularly discriminate between CAR and PKA mediated responses and to enter the cell and activate PKA *in vivo*. The structure of these compounds is presented in Fig. 1. Sp and Rp isomers carry the sulfur replacement of the exocyclic oxygen at, respectively, the axial and equatorial positions. These substitutions increase lipophilicity, which is evident by an increase of log K'_w (Braumann and Jastorff, 1985), induce resistance to hydrolysis by PDE (Van Haastert *et al.*, 1983; Eckstein, 1985; Braumann *et al.*, 1986), and influence the agonistic or antagonistic properties of the derivatives (Rothermel *et al.*, 1983; Van Haastert *et al.*, 1984, 1987; Buchler *et al.*, 1988; Adashi *et al.*, 1990). In *Dictyostelium*, the 5′-oxygen and 2′-hydroxy positions act as a hydrogen bond

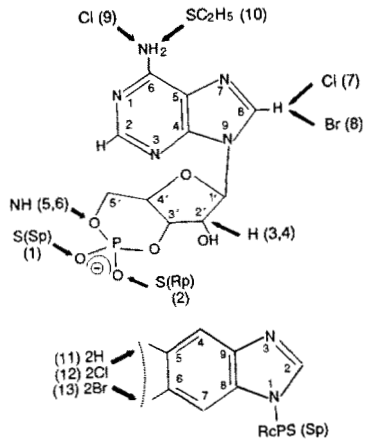

FIG. 1. **Structure of cAMP and phosphorothioate derivatives.**

acceptor and donor for binding of the molecule to PKA, but not to CAR (De Wit *et al.*, 1982; Van Haastert and Kien, 1983). In compounds 3–6 hydrogen bonding at these positions is prevented. Conversely, the N^6H_2 group is essential for hydrogen bond formation with CAR, but not with PKA. Substitution by a chloro or thioethyl group strongly reduces the affinity for CAR, has little effect on affinity for PKA, and increases membrane permeability by reducing the polarity of the molecule. The bromo and chloro substitutions at the 8-position alter the *syn/anti* equilibrium to preferably *syn*. These modifications and substitution of adenine for benzimidazole increase lipophilicity of the molecule, which is indicated by a relatively high log K'_w (Table I).

Degradation of cAMP Phosphorothioates—We first investigated the stability of phosphorothioates, by incubating 10 μM of derivative with 3×10^6 cells/ml during 4 h and measuring the levels of derivative by HPLC reverse-phase chromatography. As shown in Fig. 2 for Sp-cAMPS, Rp-cAMPS, Sp-2′HcAMPS, Sp-5′NHcAMPS, and Sp-8BrcAMPS, less than 30% of phosphorothioate is degraded during 4 h of incubation with cells. This is true for all phosphorothioates used in this study (data not shown). 10 μM cAMP has, under these conditions, a half-life of about 15 min. Ineffective degradation by PDE may be caused by inefficient binding to the enzyme (high K_m) or a low hydrolysis rate (low V_{max}). Fig. 3*C* shows displacement of [^{3}H]cAMP from binding to PDE by cAMP and five phosphorothioates. While cAMP induces half-maximal inhibition of [^{3}H]cAMP degradation at about 3 μM, Sp-cAMPS, Sp-2′HcAMPS, Sp-8BrcAMPS, and Sp-6SEtcAMPS require at least 200-fold higher concentrations. However, Sp-5′NHcAMPS requires only 10-fold higher concentrations than cAMP, but is nevertheless as slowly hydrolyzed as the other derivatives (Fig. 2). This emphasizes that the stability of phosphorothioates for degradation by PDE is not only due to their low affinity for binding to the enzyme, but also to their low intrinsic hydrolysis rate (Van Haastert *et al.*, 1983).

Binding of cAMP-phosphorothiates to CAR, PKA, and PDE—Fig. 3 shows the inhibition by non-hydrolyzable cAMP derivatives of [^{3}H]cAMP binding to CAR (*A*), PKA R-subunit (*B*), and to PDE (*C*). IC_{50} values (concentrations inducing half-maximal inhibition) calculated from dose-response curves are summarized in Table II. Replacement of the axial exocyclic oxygen by sulfur (Sp-cAMPS) reduces affinity for CAR about 80-fold, for PKA 20-fold, and for PDE 200-fold. Replacement of the equatorial oxygen (Rp-cAMPS) reduces affinities for CAR, PKA, and PDE, respectively, 300-, 170-,

TABLE I
Nomenclature of cAMP phosphorothioates

No.	Abbreviation	Name	log K_w'[a]
1	Sp-cAMPS	Adenosine 3′:5′-monophosphorothioate Sp-isomer	1.23
2	Rp-cAMPS	Adenosine 3′:5′-monophosphorothioate Rp-isomer	1.15
3	Sp-2′HcAMPS	2′-Deoxyadenosine 3′:5′-monophosphorothioate Sp-isomer	1.28
4	Rp-2′HcAMPS	2′-Deoxyadenosine 3′:5′-monophosphorothioate Rp-isomer	1.14
5	Sp-5′NHcAMPS	5′-Deoxy-5′aminoadenosine 3′:5′-monophosphorothioate Sp-isomer	0.90
6	Rp-5′NHcAMPS	5′-Deoxy-5′aminoadenosine 3′:5′-monophosphorothioate Rp-isomer	0.80
7	Sp-8ClcAMPS	8-Chloroadenosine 3′:5′-monophosphorothioate Sp-isomer	1.70
8	Sp-8BrcAMPS	8-Bromoadenosine 3′:5′-monophosphorothioate Sp-isomer	1.90
9	Sp-6ClcPUMPS	6-Chloropurineriboside 3′:5′-monophosphorothioate Sp-isomer	1.48
10	Sp-6SEtcPUMPS	6-Thioethylpurineriboside 3′:5′-monophosphorothioate Sp-isomer	2.27
11	Sp-cBIMPS	Benzimidazoleriboside 3′:5′-monophosphorothioate Sp-isomer	1.55
12	Sp-DClcBIMPS	5,6-Dichlorobenzimidazoleriboside 3′:5′-monophosphorothioate Sp-isomer	2.93
13	Sp-DBrcBIMPS	5,6-Dichlorobenzimidazoleriboside 3′:5′-monophosphorothioate Sp-isomer	3.34

[a] $\log K_w' = \log K_w$ (derivative)/($\log K_w$ (cAMP)).

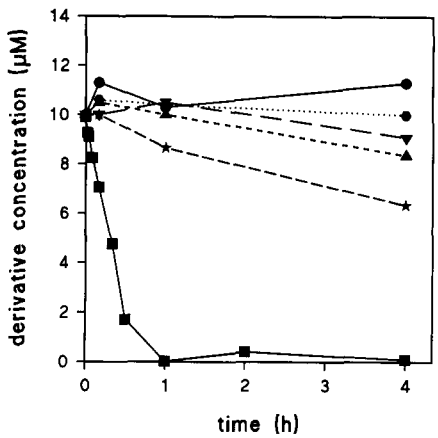

FIG. 2. **Degradation of cAMP phosphorothioates by *Dictyostelium* cells.** Aggregation competent cells were incubated at 3 × 10^6 cells/ml with 10 μM of cAMP or phosphorothioates at 20 °C. At the indicated time intervals, 20-μl samples were removed and analyzed by HPLC to measure levels of remaining derivative. ■, cAMP; ●, Sp-cAMPS; ●, Rp-cAMPS; ★, Sp-2′HcAMPS; ▲, Sp-5′NHcAMPS; ▼, Sp-8BrcAMPS.

and more than 300-fold. The additional 2′H and 5′NH modifications have little effect on binding to CAR, but reduce binding to PKA, respectively, 1800- and 300-fold compared to Sp-cAMPS. The 8-Cl and 8-Br modifications reduce affinity to CAR, respectively, 20- and 40-fold, but do not affect binding to PKA. The derivatives substituted at the 6-position, as well as the three Sp-benzimidazoles are very poor CAR agonists, but show high affinity to PKA.

Except for Rp- and Sp-5′NHcAMPS, all phosphorothioates show very low affinity for PDE. The first two derivatives show, respectively, 13 and 25 times higher affinity for PDE than Sp-cAMPS, which mirrors the 14-fold higher affinity of 5′NHcAMP for PDE, compared to cAMP (Van Haastert *et al.*, 1983).

To conclude, among the tested derivatives, compounds 3–6 are highly selective for binding to CAR, while compounds 7–13 are selective PKA agonists. All compounds are resistant to degradation by PDE.

Activation of PKA in Relation to Binding—The derivatives were also tested for their ability to activate the PKA holoenzyme. From earlier results it was concluded that modification of the exocyclic oxygen atoms may reduce the level of activation, especially with Rp-cAMPS, which acts as an antagonist of mammalian PKA (Rothermel *et al.*, 1983; Buchler *et al.*, 1988; Adashi *et al.*, 1990), and as a partial antagonist/agonist of *Dictyostelium* PKA (De Wit *et al.*, 1984). The dose-dependent activation of PKA by Sp derivatives is presented in Fig. 4. Most of the PKA-specific Sp derivatives showed reduced activation of PKA at high concentrations, which may be caused by competition of these compounds for the ATP-binding site. The calculated EC_{50} values (Table III) show that derivatives with high binding affinity for the regulatory subunit of PKA also effectively activate the PKA holoenzyme.

Uptake of cAMP Phosphorothioates—The derivatives Sp-6SEtcPuMPS and Sp-8BrcAMPS show relatively high lipophilicity and affinity for PKA. These derivatives may pass the plasmamembrane more easily and be a useful tool to activate PKA *in vivo*. To measure uptake, aggregation competent *D. discoideum* cells were incubated for 0 and 45 min with 30 μM Sp-cAMPS, Sp-8BrcAMPS, and Sp-6SEtcPuMPS, washed extensively, and then lysed. The accumulation of cell-associated derivatives was measured. Table IV shows that Sp-cAMPS poorly enters the cell. The calculated intracellular concentration is about 500-fold lower than the extracellular concentration. Sp-8BrcAMPS and Sp-6SEtcPuMPS are considerably more cell permeable. Incubation with 30 μM extracellular Sp-8BrcAMPS results in an estimated intracellular concentration of 0.5 μM. Incubation with 30 μM Sp-6SEtcPuMPS yields an intracellular concentration of about 0.8 μM. This is sufficiently high to activate intracellular PKA, whereas the extracellular concentration of 30 μM is insufficient to activate cell surface cAMP receptors.

Induction of Gene Expression by cAMP Phosphorothioates—Gene expression was measured in cell lines transformed with vectors carrying fusions of the *E. coli lac*Z gene with, respectively, the promoter of the prespore gene D19 (Dingermann *et al.*, 1989), the prestalk-enriched gene CP2 (Datta and Firtel, 1988),[4] and the promoter of the PDE gene, which directs expression in the aggregative phase of development (Faure *et al.*, 1990).[3] Promoter activity results in synthesis of the β-galactosidase enzyme, which can be detected with a highly sensitive and quantitative spectrophotometric assay. The various cell lines were incubated on non-nutrient agar until competence for induction of expression of the respective genes had been reached and were subsequently incubated in suspension in microtiter plate wells with a single dose of phosphorothioate derivative. Fig. 5 shows dose-response relationships for induction of expression of the three genes by six derivatives. Of immediate interest is the observation that the PDE and CP2 genes are induced by at least 200-fold lower concentrations than the D19 prespore gene. The CAR agonist Sp-5′NHcAMPS is as effective as Sp-cAMPS in inducing both PDE, CP2, and D19 gene expression. The PKA agonist Sp-6SEtcPUMPS cannot induce any D19 expression at concentrations up to 1 mM, while Sp-8BrcAMPS induces some D19

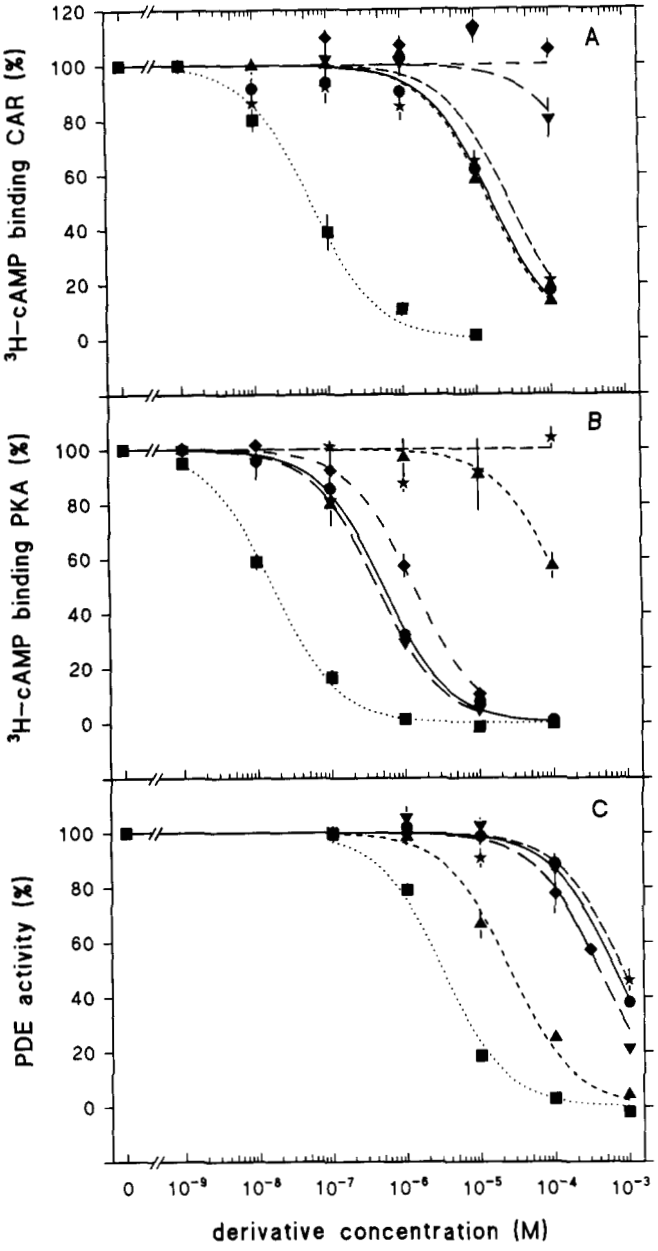

FIG. 3. **Binding of cAMP phosphorothioates to CAR, PKA, and PDE.** To measure the affinity of phosphorothioates for CAR, aggregation competent AX3 cells were incubated at 0 °C with 3 nM [^{3}H]cAMP and the indicated concentrations of derivatives. After 1 min, cells were separated from unbound [^{3}H]cAMP by centrifugation through silicon oil. Affinity for PKA regulatory subunit was determined by incubating a PKA R-subunit preparation for 90 min at 0 °C with 3 nM [^{3}H]cAMP and derivatives. Unbound radioactivity was removed by filtration. To measure the affinity of phosphorothioates for PDE, the derivatives were incubated during 30 min at 20 °C with 1 μM [^{3}H]cAMP and an ePDE preparation. After 30 min, the newly formed [^{3}H]AMP was further degraded to [^{3}H]adenosine and unhydrolyzed [^{3}H]cAMP was removed with anion-exchange resin. All data represent the mean and S.E. of at least two individual experiments performed in triplicate and are expressed as percentage of binding or degradation obtained in the absence of derivative. ■, cAMP; ●, Sp-cAMPS; ★, Sp-2′HcAMPS; ▲, Sp-5′NHcAMPS; ▼, Sp-8BrcAMPS; ◆, Sp-6SEtcAMPS.

expression. Both agonists are also very poor inducers of PDE and CP2 expression.

Surprisingly, the CAR antagonist Rp-cAMPS, which does not activate cAMP-regulated second messenger responses (Van Haastert *et al.*, 1984, 1987), can induce expression of the PDE and CP2 gene. However, concentrations required for half-maximal induction are about 20 times higher than expected from its affinity for CAR. Rp-cAMPS does not induce any D19 expression at concentrations up to 1 mM. Of further interest is the fact that the CAR agonist Sp-2′HcAMPS is a relatively poor inducer of all three types of gene expression.

Comparison of Binding and Gene Induction Data—EC_{50} values (concentrations required for half-maximal activation) for induction of the three different genes by all selected phosphorothioate derivatives are summarized in Table V and compared with IC_{50} values for binding of the derivatives to CAR, PKA, and PDE in Fig. 6. These data show that for all the three genes the highly selective PKA agonists Sp-6ClcPUMPS, Sp-6SEtcPUMPS, Sp-DClcBIMPS, and Sp-DBrcBIMPS are virtually ineffective to induce expression. The other PKA agonists Sp-8BrcAMPS and Sp-8ClcAMPS can induce low levels of gene expression at very high concentrations. However, these compounds also bind moderately well to CAR. The CAR agonist Sp-5′NHcAMPS induces expression of PDE, CP2, and D19 gene expression very efficiently. These specificity profiles strongly suggest that induction of gene expression is mediated by CAR and not by PKA.

The Rp-isomers of cAMPS and 5′NHcAMPS can induce PDE and CP2 gene expression, however, induction is considerably less efficient than predicted by the relative affinity of the Rp-isomers for surface cAMP receptors. The Rp-isomer of 2′HcAMPS is ineffective and mirrors the unexpected low efficiency of induction by its Sp counterpart. D19 gene expression cannot be induced by any of the Rp-isomers.

DISCUSSION

In this report we have investigated the properties of non-hydrolyzable cAMP phosphorothioates, designed to activate intracellular PKA in *Dictyostelium* and to discriminate between PKA- and CAR-mediated responses. Several derivatives were identified that show high selectivity for PKA relative to CARs and vice versa. The PKA agonists also bind effectively to cAMP-dependent protein kinase from mammalian cells (Dostmann *et al.*, 1990). *Dictyostelium* cells are generally not very permeable for charged compounds. The more lipophilic cAMP derivatives were, however, shown to penetrate the cells reaching intracellular concentrations that are sufficient to activate PKA. These derivatives should be very useful in unraveling the function of intracellular cAMP and PKA.

The cAMP phosphorothioates were used to investigate involvement of either CARs or PKA in control of expression of three classes of cAMP-regulated genes. Nucleotide specificity of induction of prestalk and prespore gene expression has been investigated earlier, using cAMP derivatives with unmodified exocyclic oxygen atoms, which have the distinct disadvantage of being rapidly hydrolyzed by the cells during the prolonged incubation periods required for gene induction. Since not all these derivatives are equally sensitive to degradation and the resulting metabolites may have unpredictable side effects, these studies cannot yield accurate dose-response relationships and may lead to erroneous conclusions. An important example of such misinterpretation is provided by studies of proliferation inhibition by the putative anticancer drug 8-chloro-cAMP, which was supposedly mediated solely by PKA (Ally *et al.*, 1989) but was recently shown to be largely induced by the metabolite 8-chloroadenosine, which must be acting by an entirely different mechanism (Van Lookeren Campagne *et al.*, 1991).

We show here that induction of the prestalk gene CP2, the prespore gene D19, and PDE gene expression, controlled by

TABLE II

Binding of cAMP phosphorothioates to three cAMP-binding proteins

IC_{50} values (concentrations inducing half-maximal inhibition of [^{3}H]cAMP binding or [^{3}H]cAMP degradation) were calculated from dose-response curves by fitting the curve to the function for first order ligand binding. Means and S.E. of at least two independent dose-response measurements are presented.

No.	Derivative	CAR	PKA	PDE
			IC_{50} (M)	
	cAMPS	$2.4 \times 10^{-7} \pm 9.3 \times 10^{-8}$	$2.8 \times 10^{-8} \pm 7.3 \times 10^{-9}$	$3.1 \times 10^{-6} \pm 4.5 \times 10^{-7}$
1	Sp-cAMPS	$2.0 \times 10^{-5} \pm 2.7 \times 10^{-6}$	$5.6 \times 10^{-7} \pm 1.1 \times 10^{-7}$	$6.4 \times 10^{-4} \pm 2.3 \times 10^{-5}$
2	Rp-cAMPS	$7.3 \times 10^{-5} \pm 0.9 \times 10^{-6}$	$4.9 \times 10^{-6} \pm 2.1 \times 10^{-6}$	$1.0 \times 10^{-3} \pm 1.4 \times 10^{-5}$
3	Sp-2′HcAMPS	$2.9 \times 10^{-5} \pm 9.3 \times 10^{-6}$	$>10^{-3}$	$8.7 \times 10^{-4} \pm 1.7 \times 10^{-4}$
4	Rp-2′HcAMPS	$8.9 \times 10^{-5} \pm 1.5 \times 10^{-5}$	$>10^{-3}$	$1.1 \times 10^{-3} \pm 6.4 \times 10^{-5}$
5	Sp-5′NHcAMPS	$1.9 \times 10^{-5} \pm 2.5 \times 10^{-7}$	$1.5 \times 10^{-4} \pm 3.8 \times 10^{-5}$	$2.7 \times 10^{-5} \pm 2.1 \times 10^{-6}$
6	Rp-5′NHcAMPS	$9.6 \times 10^{-5} \pm 1.0 \times 10^{-5}$	$1.7 \times 10^{-4} \pm 7.0 \times 10^{-5}$	$5.2 \times 10^{-5} \pm 6.9 \times 10^{-6}$
7	Sp-8ClcAMPS	$3.5 \times 10^{-4} \pm 1.2 \times 10^{-4}$	$7.0 \times 10^{-7} \pm 8.1 \times 10^{-8}$	$9.2 \times 10^{-4} \pm 4.6 \times 10^{-6}$
8	Sp-8BrcAMPS	$7.6 \times 10^{-4} \pm 1.6 \times 10^{-4}$	$4.2 \times 10^{-7} \pm 1.7 \times 10^{-8}$	$3.9 \times 10^{-4} \pm 2.2 \times 10^{-5}$
9	Sp-6ClcPUMPS	$>10^{-2}$	$1.3 \times 10^{-6} \pm 2.1 \times 10^{-8}$	$1.4 \times 10^{-3} \pm 4.4 \times 10^{-4}$
10	Sp-6SEtcPUMPS	$>10^{-2}$	$1.3 \times 10^{-6} \pm 4.9 \times 10^{-7}$	$3.9 \times 10^{-4} \pm 4.7 \times 10^{-5}$
11	Sp-cBIMPS	$>10^{-2}$	$5.1 \times 10^{-6} \pm 8.3 \times 10^{-7}$	$7.8 \times 10^{-4} \pm 3.2 \times 10^{-6}$
12	Sp-DClcBIMPS	$>10^{-2}$	$1.3 \times 10^{-6} \pm 2.6 \times 10^{-7}$	$1.9 \times 10^{-4} \pm 2.3 \times 10^{-5}$
13	Sp-DBrcBIMPS	$>10^{-2}$	$1.7 \times 10^{-6} \pm 2.6 \times 10^{-7}$	$2.0 \times 10^{-4} \pm 1.5 \times 10^{-5}$

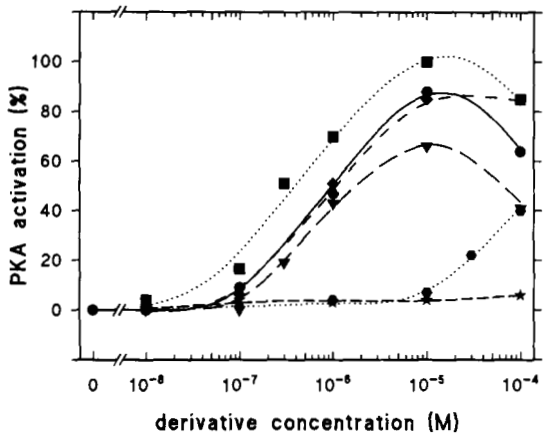

FIG. 4. **Activation of *D. discoideum* PKA by cAMP phosphorothioates.** Partially purified PKA from *D. discoideum* slugs was used to measure activation of PKA holoenzyme by phosphorothioate derivatives as described under "Experimental Procedures." Data are expressed as percentage of PKA activation induced by 10^{-5} M cAMP. Results represent the means of two experiments performed in triplicate. ■,: cAMP; ●, Sp-cAMPS; ●, Rp-cAMPS; ★, Sp-2′HcAMPS; ▼, Sp-8BrcAMPS; ♦, Sp-6SEtcAMPS.

TABLE III

Activation of PKA from Dictyostelium by cAMP phosphorothioates

No.	Derivative	PKA activation
		EC_{50} (M)[a]
	cAMP	1.1×10^{-7}
1	Sp-cAMPS	1.6×10^{-6}
3	Sp-2′HcAMPS	$>1.0 \times 10^{-4}$[b]
7	Sp-8ClcAMPS	2.0×10^{-6}
8	Sp-8BrcAMPS	1.5×10^{-6}
9	Sp-6ClcPUMPS	3.0×10^{-6}
10	Sp-6SEtcPUMPS	1.1×10^{-6}

[a] The EC_{50} values were retrieved from Eadie-Hofstee plots (see Fig. 4 for primary data).

[b] No activation observed (the derivative does not bind to PKA; see Table II).

the aggregative promoter, show a specificity profile for phosphorothioates similar to the affinity profile of CAR and entirely distinct from the affinity profile of PKA. The membrane-permeable derivatives Sp-6SEtcPUMPS and Sp-8BrcAMPS cannot activate CP2 or D19 expression at concentrations, which are 10-fold higher than required for PKA activation. This observation not only rules out that PKA is the first target for cAMP regulation of gene expression, but also contradicts a function for intracellular cAMP as downstream component of the transduction pathway of extracellular cAMP.

TABLE IV

Uptake of cAMP phosphorothioates

Derivative	Cell-associated derivative	Intracellular conc[a]	Extracellular/ intracellular
	pmol/2.7 × 10^7 cells	*μM*	
Sp-cAMPS	0.45	0.063	480
Sp-8BrcAMPS	3.55	0.49	61
Sp-6SEtcPUMPS	5.93	0.82	36

[a] It is assumed that 2.7×10^7 cells have a volume of 7.2 μl (cell radius 4 μm). 2.7×10^7 aggregation competent *Dictyostelium* cells were incubated with 30 μM of the indicated phosphorothioates. After 0 and 45 min, the cells were washed extensively, lysed with perchloric acid, and neutralized in $KHCO_3$. The concentration of cell-associated derivative was measured with a cAMP-binding protein isotope dilution assay as described under "Experimental Procedures." Control experiments showed that the cross-reacting compound(s) from cells that were incubated for 0 min with cAMP phosphorothioates and then washed could be completely degraded by PDE. This indicates that endogenous cAMP was detected and that all added phosphorothioates were removed effectively. In contrast, the cross-reacting compound(s) from cells incubated for 45 min with phosphorothioates were largely non-degradable by PDE, indicating the association of phosphorothioate with the cells.

Our present evidence against involvement of PKA in cAMP-induced D19 and CP2 expression agrees with earlier data that expression of these genes in response to cAMP is normal under conditions where adenylyl cyclase cannot be activated (Schaap *et al.*, 1986; Bozzaro *et al.*, 1987; Mann *et al.*, 1988). However, they are partly in contradiction with recent studies claiming universal importance of PKA in prespore and prestalk gene regulation. Inactivation of PKA by overexpression of native or dominant negative R-subunit blocks development and inhibits expression of the cAMP-induced genes CP2 and D19 and the *ecmA* and *ecmB* genes, induced by the differentiation inducing factor, DIF (Simon *et al.*, 1989; Firtel and Chapman, 1990; Harwood *et al.*, 1992a, 1992b). cAMP can furthermore not induce prespore gene expression in null mutants for the PKA catalytic subunit (Mann and Firtel, 1991).[2] Additional evidence for the ubiquitous importance of PKA is implicated by rapid development and precocious expression of stalk and spore genes in *rdeC*

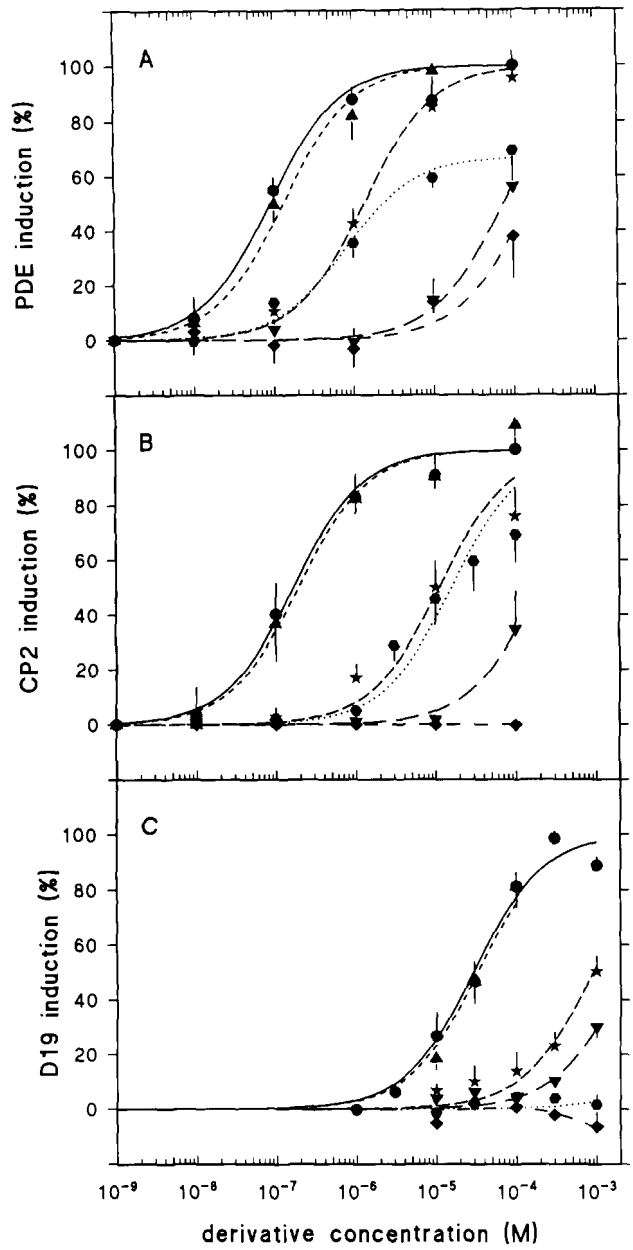

FIG. 5. **Induction of three classes of cAMP regulated genes by phosphorothioates.** Vegetative PDE_{ag}-*lacZ* cells, CP2-*lacZ* cells starved for 4 h at 20 °C and D19-*lacZ* cells starved for 16 h at 6 °C were incubated in PB at 3×10^6 cells/ml and 20 °C with the indicated phosphorothioate concentrations. After 6 h cells were lysed by freeze-thawing, and β-galactosidase activity was determined. Data represent mean and S.E. from at least three individual experiments performed in duplicate. ●, Sp-cAMPS; ●, Rp-cAMPS; ★, Sp-2′HcAMPS; ▲, Sp-5′NHcAMPS; ▼, Sp-8BrcAMPS; ♦, Sp-6SEtcAMPS.

mutants, which lack the PKA R-subunit (Simon *et al.*, 1992), and in transformants overexpressing the PKA catalytic subunit (Anjard *et al.*, 1992).

The DIF-induced genes and the late spore genes were not investigated in the present study and may well be regulated by PKA activation. In the case of the PDE gene, our data neither contradict nor support an inhibitory role for PKA in its expression. However, since PDE gene expression is under positive control of extracellular cAMP, intracellular cAMP cannot be an intermediate of the extracellular signal. The gene is possibly controlled by other extracellular or cell autonomous signals involving modulation of intracellular cAMP levels.

A strong discrepancy concerns involvement of PKA in CP2 and D19 regulation. One explanation could be that CP2 and D19 gene expression require constitutive activity of PKA. Compared to mammalian PKA, the regulatory and catalytic subunits of *Dictyostelium* PKA are rather loosely associated (De Gunzburg *et al.*, 1984), and the *Dictyostelium* enzyme could be active in the absence of cAMP. Since disruption of the catalytic subunit gene, or overexpression of R-subunit, would inactivate all available catalytic subunits, this could explain inhibition of gene expression in these transformants.

The importance of resistance to degradation for adequate estimation of dose-dependence is evident by the rather unexpected efficacy of Sp-cAMPS to induce CP2 gene expression. It was estimated earlier that cAMP induces half-maximal induction of this gene around 15 μM (Mehdy and Firtel, 1985). Sp-cAMPS induces half-maximal expression around 100 nM, which means that cAMP is actually active at steady state concentrations between 1 and 5 nM. Much higher cAMP concentrations are required to counteract degradation, which gives a misleading impression of involvement of low affinity receptors. D19 induction occurs half-maximally at 30 μM Sp-cAMPS, which agrees with an earlier estimate of the EC_{50} for cAMP around 1 μM, which was corrected for degradation by PDE (Schaap and Van Driel, 1985; Oyama and Blumberg, 1986).

An unexpected outcome of present experiments is the fact that Rp-cAMPS can induce PDE and CP2 gene expression. This compound was previously described as a chemotactic antagonist of cAMP, *i.e.* it binds to, but does not activate chemotactic receptors and inhibits chemotaxis induced by cAMP. Rp-cAMPS can furthermore not activate adenylyl cyclase or guanylyl cyclase (Van Haastert *et al.*, 1984, 1987). Rp-cAMPS activation of phospholipase C has not yet been studied, but since the less potent antagonist 8-*p*-chlorophenylthio-cAMP cannot activate this enzyme (Peters *et al.*, 1991), it is probably also not activated by Rp-cAMPS. Since Rp-cAMPS can induce PDE and CP2 gene expression, this suggests that neither cAMP, cGMP, nor $InsP_3$/diacylglycerol mediate ligand-induced expression of these genes. However, it cannot be completely ruled out that Rp-cAMPS may function as a very poor partial antagonist, inducing some response at supersaturating concentrations.

Absolute EC_{50} values for binding of cAMP and SpcAMPS to surface receptors measured in the present study are about 10-fold higher than previously reported (Van Ments-Cohen and Van Haastert, 1989), and may therefore represent binding to the low affinity form of surface receptors rather than the high affinity form. This is probably due to the fact that in this study we measured binding to AX3 cells, instead of NC4 cells used in the previous studies. AX3 cells show considerably stronger oscillatory cAMP signaling in suspension, and the increased cAMP levels would bring the majority of receptors in the low affinity state. Since both affinity states have the same analog specificity profile (Van Ments-Cohen and Van Haastert, 1989), this does not influence the relative potency of the phosphorothioates to bind to surface receptors. The specificity profile of phosphorothioates for binding to CAR, measured in aggregation-competent cells, most likely represents binding to CAR1, since this is by far the most abundant binding activity at this stage. CAR2, CAR3, and CAR4 have too little binding activity to allow specificity analysis in wild type cells. The nucleotide specificity for oxo-cAMP derivatives of three receptors (CAR1, CAR2, and CAR3) was recently determined in transformants overexpressing the three receptors in the vegetative stage (Johnson *et al.*, 1992). The specificity profile of the three receptors was highly similar,

TABLE V

EC_{50} values for induction of three classes of cAMP regulated genes by cAMP phosphorothioates

Means and S.E. of EC_{50} values (concentrations yielding half-maximal induction of gene expression) derived from three to six independent measurements of dose response relationships for gene induction are presented.

No.	Derivative	PDE_{ag} induction	CP2 prestalk induction	D19 prespore induction
			EC_{50} (M)	
1	Sp-cAMPS	$1.2 \times 10^{-7} \pm 2.3 \times 10^{-8}$	$2.2 \times 10^{-7} \pm 5.6 \times 10^{-8}$	$2.9 \times 10^{-5} \pm 4.1 \times 10^{-6}$
2	Rp-cAMPS	$1.3 \times 10^{-5} \pm 6.9 \times 10^{-6}$	$1.8 \times 10^{-5} \pm 4.1 \times 10^{-6}$	$>10^{-2}$
3	Sp-2′HcAMPS	$2.4 \times 10^{-6} \pm 5.7 \times 10^{-7}$	$1.5 \times 10^{-5} \pm 4.9 \times 10^{-6}$	$1.1 \times 10^{-3} \pm 4.2 \times 10^{-4}$
4	Rp-2′HcAMPS	$1.2 \times 10^{-4} \pm 2.4 \times 10^{-5}$	$8.2 \times 10^{-4} \pm 1.3 \times 10^{-4}$	$>10^{-2}$
5	Sp-5′NHcAMPS	$1.3 \times 10^{-7} \pm 4.0 \times 10^{-8}$	$2.9 \times 10^{-7} \pm 7.7 \times 10^{-8}$	$3.3 \times 10^{-5} \pm 5.1 \times 10^{-6}$
6	Rp-5′NHcAMPS	$1.1 \times 10^{-5} \pm 2.7 \times 10^{-6}$	$1.7 \times 10^{-5} \pm 4.0 \times 10^{-5}$	$>10^{-2}$
7	Sp-8ClcAMPS	$3.2 \times 10^{-5} \pm 6.8 \times 10^{-6}$	$3.5 \times 10^{-4} \pm 1.3 \times 10^{-4}$	$>10^{-2}$
8	Sp-8BrcAMPS	$5.5 \times 10^{-5} \pm 3.2 \times 10^{-5}$	$4.7 \times 10^{-4} \pm 2.8 \times 10^{-4}$	$6.0 \times 10^{-3} \pm 3.6 \times 10^{-3}$
9	Sp-6ClcPUMPS	$2.5 \times 10^{-5} \pm 1.7 \times 10^{-6}$	$1.6 \times 10^{-4} \pm 1.4 \times 10^{-6}$	$>10^{-2}$
10	Sp-6SEtcPUMPS	$4.5 \times 10^{-5} \pm 1.0 \times 10^{-5}$	$>10^{-3}$	$>10^{-2}$
11	Sp-cBIMPS	$5.2 \times 10^{-5} \pm 7.2 \times 10^{-6}$	$8.3 \times 10^{-5} \pm 2.3 \times 10^{-5}$	$1.7 \times 10^{-3} \pm 7.5 \times 10^{-4}$
12	Sp-DClcBIMPS	$6.1 \times 10^{-4} \pm 2.3 \times 10^{-4}$	$>10^{-3}$	$>10^{-2}$
13	Sp-DBrcBIMPS	$1.6 \times 10^{-4} \pm 5.0 \times 10^{-6}$	$>10^{-3}$	$>10^{-2}$

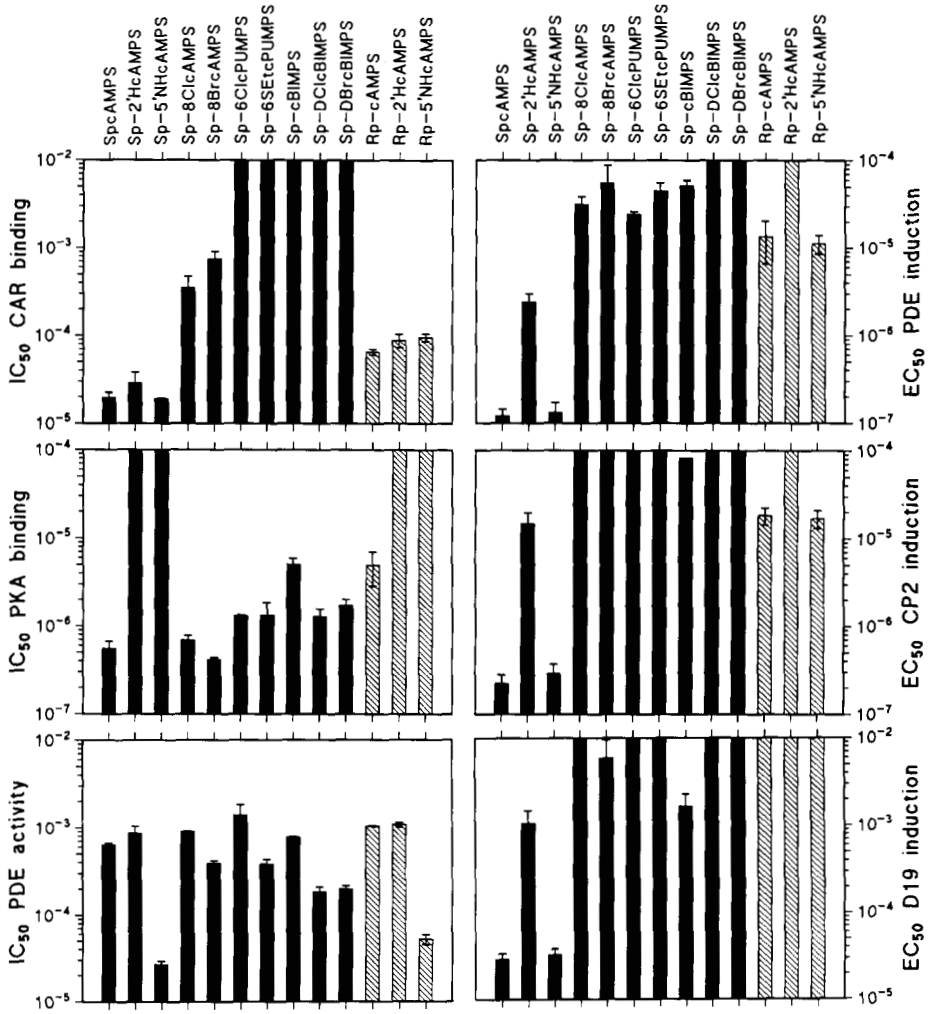

FIG. 6. **Specificity profiles of three cAMP-binding proteins and three classes of cAMP-regulated genes.**

although CAR2 and CAR3 show increased stereo-selectivity for the axial exocyclic oxygen. All CARs show two affinity states in the 4–30 nM and 200–500 nM concentration range.

Some evidence arguing for involvement of other CARs than CAR1 in gene induction is provided by the observation that Sp-2′HcAMPS is 10–50-fold less effective for inducing expression of the three classes of genes than expected from its affinity for CAR1. This in contrast to 2′HcAMP, which induces gene expression as predicted by its relative affinity for CAR1 (Van Haastert and Kien, 1983; Schaap and Van Driel, 1985; Oyama and Blumberg, 1986; Haribabu and Dottin, 1986; Gomer *et al.*, 1986). In addition, Sp-8ClcAMPS and Sp-8BrcAMPS are less efficient inducers of gene expression than expected from their affinity for CAR1. Possibly, these derivatives and Sp-2′HcAMPS show reduced affinity for one of the other CARs, which would then be a good candidate to mediate gene induction.

Acknowledgments—We are grateful to Bas Slierendregt for per-

forming part of the binding experiments and to Peter Michielsen and Joost Storms for measuring degradation of phosphorothioates. We also thank Drs. Anne L. Hall and Richard H. Kessin for their kind gift of the PDE_{sg}-*lacZ* transformed cell line, Dr. Jeffrey G. Williams for providing the D19-*lacZ* transformed cell line, and Christopher Gaskins for preparing the CP2-*lacZ* transformation vector.

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