Selective induction of gene expression and second-messenger accumulation in Dictyostelium discoideum by the partial chemotactic antagonist 8-p-chlorophenylthioadenosine 3',5'-cyclic monophosphate

(cAMP derivatives/insitolphospholipid signaling/GTP-binding protein/gene regulation/transmembrane signal transduction)

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ABSTRACT During development of the cellular slime mold Dictyostelium discoideum, cAMP induces chemotaxis and expression of different classes of genes by means of interaction with surface cAMP receptors. We describe a cAMP derivative, 8-p-chlorophenylthioadenosine 3',5'-cyclic monophosphate (8-CPT-cAMP), which inhibits cAMP-induced chemotaxis at low concentrations but induces chemotaxis at supersaturating concentrations. This compound, moreover, selectively activates expression of aggregative genes but not of postaggregative genes. 8-CPT-cAMP induces normal cGMP and cAMP accumulation but in contrast to cAMP, which increases inositol 1,4,5-trisphosphate levels, 8-CPT-cAMP decreases inositol 1,4,5-trisphosphate levels. The derivative induces reduced activation of guanine nucleotide regulatory proteins, which may cause its defective activation of inositol 1,4,5-trisphosphate production. Our data suggest that disruption of insitolphospholipid signaling impairs chemotaxis and expression of a subclass of cAMP-regulated genes.

In the social amoebae Dictyostelium discoideum, extracellular cAMP functions as a hormone-like signal; it induces the expression of several classes of genes and regulates morphogenetic movement by acting as a chemoattractant (see ref. 1). cAMP signal processing is very similar to that of mammalian hydrophilic hormones, such as adrenaline, vasopressin, acetylcholine, luteinizing hormone, and many others; its effects on chemotaxis and gene expression are mediated by surface receptors (2–6), which belong to the ubiquitous class of seven-trans-membrane receptors, interacting with guanine nucleotide regulatory protein (G) proteins (7, 8). This interaction results in activation of target enzymes, such as adenylate cyclase, guanylate cyclase, and phospholipase C (see ref. 9). Similar to the adrenergic receptor, for example, cAMP receptors are encoded by a family of different genes, of which three members have been cloned (10). Also, the Dictyostelium G proteins belong to a multigene family (11). Elucidation of signal-transduction cascades involved in gene regulation and chemotaxis is of crucial importance for our general understanding of these processes. By using mutants and molecular genetic approaches, considerable progress has been made in understanding some of the functional relations between cAMP receptors, G proteins, second-messenger systems, and the ultimate responses that they control (7, 11–17). We describe here a modified cAMP receptor ligand, 8-p-chlorophenylthioadenosine 3',5'-cyclic monophosphate (8-CPT-cAMP), which may be a powerful pharmacological tool for dissecting cAMP transduction cascades. This cAMP derivative selectively activates some second-messenger systems, a subpopulation of G proteins, and a subpopulation of cAMP-regulated genes.

MATERIALS AND METHODS

Materials. Luciferin and guanosine 5'-[γ-thio]triphosphate GTP[yS] were from Boehringer Mannheim. 8-CPT-cAMP was supplied by B. Jastorff (University of Bremen, Bremen, F.R.G.) or purchased from Boehringer Mannheim. 8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP), 6-chloropurinoriboside 3',5'-cyclic monophosphate (6-Cl-cUMP), o-nitrophenyl β-D-galactoside, Geneticin (G418), and phenylmethylsulfonyl fluoride were obtained from Sigma, [2,8-3H]cAMP, [α-32P]dATP, and cGMP RIA kits were from Amersham, and GTP[γS] was from New England Nuclear.

Dictyostelium Strains and Culture Conditions. D. discoideum strain NC-4 and mutant snaq 7 (18) were grown on glucose/peptone agar in association with Escherichia coli 281. Two transformed axenic (AX2) cell lines (D19–lacZ and CP2–luciferase) were grown in HL5 medium (19) in the presence of G418 at 10 μg/ml. D19–lacZ cells contain the vector pA6PTlac.1, which bears a gene fusion of D19 promoter and lacZ (20); CP2–luciferase cells contain the vector PB10.act.15.BKH.LUC.BAM, which carries a fusion of the firefly luciferase gene and CP2 promoter (21).

Growing cells were freed from nutrients by repeated washing with 10 mM Na/K phosphate, pH 6.5 [phosphate buffer (PB)]. Aggregation competence was induced by incubating cells on PB agar at 2.5 × 10⁶ cells per cm² for 16 hr at 6°C or by stimulating cells for 4 hr with 30 nM cAMP pulses at 6-min intervals.

Binding and Phosphodiesterase (PDE) Assays. The affinity of 8-CPT-cAMP for cAMP-dependent protein kinase and its apparent Km and Vmax for cAMP PDE were determined by described methods (22, 23). The effects of cAMP, 8-Br-cAMP, and 8-CPT-cAMP on GTP[y35S]-binding to membranes of aggregation-competent cells were measured, as described by Snaar-Jagalska et al. (24).

Abbreviations: G protein, guanine nucleotide regulatory protein; PDE, cAMP phosphodiesterase; cAK, cAMP-dependent protein kinase; cAK, cAMP receptor; CSA, contact sites A; InsP3, inositol 1,4,5-trisphosphate; GTP[S], guanosine 5'-(γ-thio)triphosphate; 8-CPT-cAMP, 8-p-chlorophenylthioadenosine 3',5'-cyclic monophosphate; 8-Br-cAMP, 8-bromoadenosine 3',5'-cyclic monophosphate; 6-Cl-cUMP, 6-chloropurinoriboside 3',5'-cyclic monophosphate; PB, phosphate buffer.

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Table 1. Binding characteristics of cAMP derivatives

<table>
<thead>
<tr>
<th></th>
<th>K'_d of surface cAMP-binding sites</th>
<th>K'_d of cAR</th>
<th>PDE</th>
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<tbody>
<tr>
<td></td>
<td>A^H</td>
<td>A^L</td>
<td>B</td>
</tr>
<tr>
<td>cAMP Derivative</td>
<td>60*</td>
<td>450*</td>
<td>15*</td>
</tr>
<tr>
<td>8-CPT-cAMP</td>
<td>280†</td>
<td>250†</td>
<td>230†</td>
</tr>
<tr>
<td>8-Br-cAMP</td>
<td>220†</td>
<td>160†</td>
<td>130†</td>
</tr>
<tr>
<td>6-Cl-cPUMP</td>
<td>2500*</td>
<td>2200*</td>
<td>1400*</td>
</tr>
</tbody>
</table>

K'_d, K'_g of derivative/K'_g of cAMP; V', V' max of derivative/V' max of cAMP; K'_m, K'_s of derivative/K'_m of cAMP. K'_d values (in nM) of cAMP binding to the different receptors are indicated in parentheses.

*Data are derived from ref. 35.
†Data are derived from ref. 34.
‡Data are derived from ref. 22.
§Data are derived from ref. 23.

Analysis of mRNA Levels, lacZ, and Luciferase Gene Expression. Total cellular RNA was isolated from 2.5 × 10^7 cells, purified, size-fractionated on 1.5% agarose gels containing 2.2 M formaldehyde, and transferred to nylon membranes (25). RNA transfer was hybridized to 32P-labeled cDNAs, according to standard procedures (26). β-Galactosidase activity in cell lines transformed with D19-lacZ constructs was measured essentially as described by Dingerman et al. (20). To measure luciferase activity, cells were lysed with 10 µl of lysis buffer A [8 mM MgCl2/1 mM EDTA/1 mM dithiothreitol/1% Triton X-100/15% (vol/vol) glycerol/0.5 mM phenylmethylsulfonyl fluoride in 100 mM potassium phosphate, pH 7.5]. Subsequently 100 µl of 2% bovine serum albumin in lysis buffer A was added to the lysate. Reactions were started by adding 15 µl of 0.86 mM luciferin in 0.14 mM ATP to 25 µl of cell lysate (27). Chemoluminescence was measured by using the single photon-counting facility of an LKB model 1218 liquid-scintillation counter.

cGMP, cAMP, and Inositol 1,4,5-trisphosphate (InsP3) Responses. To measure cGMP responses, aliquots of 10^7 cells per ml were stimulated with cAMP or 8-CPT-cAMP in the presence of 2 mM dithiothreitol. After 0 or 10 s, incubation was terminated by adding an equal volume of 3.5% (vol/vol) perchloric acid, and cGMP levels were measured in neutralized extracts by RIA.

cAMP responses were induced by stimulating 27-µl aliquots of 2 × 10^6 cells per ml at 0°C with 3 µl of cAMP or 8-CPT-cAMP in 5 mM dithiothreitol. After 0 or 4 min of stimulation, 1.5 ml of ice-cold PB was added, cells were centrifuged for 5 s at 10,000 × g, supernatant was removed, and pellets were lysed in 30 µl of 3.5% perchloric acid. cAMP levels were measured by competition with [3H]cAMP for binding to aggregation-competent D. discoideum cells, using the ammonium sulfate stabilization assay (28).

To measure agonist-induced InsP3 accumulation, cells were resuspended in 40 mM Hepes, pH 6.5, to 5 × 10^6 cells per ml and stimulated with cAMP or 8-CPT-cAMP. At 4-s intervals, 30-µl aliquots were added to equal volumes of 3.5% perchloric acid. InsP3 levels were determined by isotopodilution assay (29).

RESULTS

Binding of 8-CPT-cAMP to cAMP Receptors and Induction of Chemotaxis. D. discoideum cells exhibit several kinetically distinct classes of surface cAMP-binding sites (30, 31), an intracellular cAMP-dependent protein kinase (cAK) (32) and a cAMP-specific PDE (33). Surface-binding sites can be distinguished as rapidly dissociating A^H and A^L sites, slowly dissociating B sites (30, 31), and a putative third class—the low-affinity C sites, which, in contrast to A and B sites, are resistant to downregulation by cAMP (34). The relative affinity of 8-CPT-cAMP, 8-Br-cAMP, and 6-Cl-cPUMP for all these binding sites is summarized in Table 1. Both 8-CPT-cAMP and 8-Br-cAMP are good cAK agonists; these agents bind to A and B sites with ~200-fold lower affinity than does cAMP and to C sites with ~50-fold lower affinity. Degradation by PDE is similar compared with cAMP. 6-Cl-cPUMP binds well to cAK but binds to all surface cAMP-binding sites with >1000-fold lower affinity than does cAMP.

Chemotaxis of aggregative D. discoideum cells to 8-CPT-cAMP and cAMP was compared by using the small population assay (36). Fig. 1 shows that cAMP induces a half-maximal chemotactic response at ~3 nM, and 8-CPT-cAMP induces the same level response at 50 µM. This concentration is ~80-fold higher than expected from the relative affinity of 8-CPT-cAMP for surface receptors. Most surprisingly, at lower concentrations (0.1–10 µM), 8-CPT-cAMP antagonizes chemotaxis induced by cAMP. Apparently, at low concentrations 8-CPT-cAMP acts as an antagonist of cAMP, and at high concentrations it acts as an agonist.

Induction of Gene Expression by 8-CPT-cAMP. The expression of aggregative genes coding for cAMP receptors (cAR), for example, and contact sites A (csA) can be effectively induced by nanomolar cAMP pulses (7, 37–39). Fig. 2 shows that in wild-type NC4 cells, 8-CPT-cAMP pulses are almost as effective as cAMP pulses at inducing cAR and csA gene expression. However, because 8-CPT-cAMP can induce cAMP relay (see Fig. 6), this result may be due to 8-CPT-cAMP-induced cAMP production. In mutant synag 7, which is deficient in adenylate cyclase activation (15, 18), induction of csA and cAR gene expression by 8-CPT-cAMP requires

Fig. 1. Induction of chemotaxis. Aggregation-competent NC-4 cells were deposited as 0.1-µl droplets of 10^7 cells per ml on hydrophobic agar. Droplets of the same volume of different concentrations of cAMP (●), 8-CPT-cAMP (●), or a combination of 10^-8 M cAMP with different concentrations of 8-CPT-cAMP (●) were placed close to the cell droplets. Each concentration was tested on 20 small populations. Every 15 min the number of droplets showing a positive response was scored. Means and SEMs derived from four experiments are presented.
half-maximal by incubating

cAMP up to mRNA levels (41).

expression of cAMP, 8-CPT-cAMP, 8-Br-cAMP, molar cAMP derivatives.

cAMP, 8-CPT-cAMP, 8-Br-cAMP, and 6-Cl-cPUMP on expression of the prespore gene D19 (40) and the postaggregative gene CP2, which is preferentially expressed in prestalk cells (41). cAMP induces half-maximal expression of both genes at \( \approx 30 \mu M \); expression is also induced by 1 mM of 6-Cl-cPUMP and 8-Br-cAMP, but no increase of D19 or CP2 mRNA levels was detectable at 8-CPT-cAMP concentrations up to 1 mM.

Dose–response relationships measured during prolonged incubation of cells with cAMP derivatives do not reflect true affinities of the cAMP-binding proteins because considerable degradation by PDE occurs during the incubation. Degradation of cAMP and cAMP derivatives by PDE can be reduced by incubating cells at low cell density. With transformants carrying promoter–reporter gene constructs, cell density could be reduced 10- to 20-fold (Fig. 4). At low cell density, half-maximal activation of the CP2 and D19 promoter is induced by 2 \( \mu M \) cAMP or 50 \( \mu M \) 6-Cl-cPUMP, whereas 8-CPT-cAMP induces very low levels of expression at 1 mM.

**Fig. 2.** Induction of aggregative gene expression. Vegetative NC4 and synag (sng) 7 cells were incubated at \( 5 \times 10^6 \) cells per ml in PB and stimulated with the indicated concentrations of cAMP or 8-CPT-cAMP at 6-min intervals. mRNA was isolated after 3 hr of incubation. Northern (RNA) blots were probed with csA and cAR1 cDNAs.

10- to 100-fold higher concentrations than induction by cAMP. This dose dependency agrees with the relative affinity of 8-CPT-cAMP for surface cAMP-binding sites and indicates that 8-CPT-cAMP is a full agonist for aggregative gene expression.

Postaggregative genes are expressed in response to micromolar cAMP concentrations (4, 5). Fig. 3 shows the effect of cAMP, 8-CPT-cAMP, 8-Br-cAMP, and 6-Cl-cPUMP on expression of the prespore gene D19 (40) and the postaggregative gene CP2, which is preferentially expressed in prestalk cells (41). cAMP induces half-maximal expression of both genes at \( \approx 30 \mu M \); expression is also induced by 1 mM of 6-Cl-cPUMP and 8-Br-cAMP, but no increase of D19 or CP2 mRNA levels was detectable at 8-CPT-cAMP concentrations up to 1 mM.

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**Fig. 3.** Induction of prespore and prestalk gene expression. Aggregation-competent cells were resuspended to \( 10^7 \) cells per ml in PB and stimulated with the indicated concentrations of cAMP, 8-Br-cAMP, 6-Cl-cPUMP, or 8-CPT-cAMP at 60-min intervals. mRNA was isolated after 3 or 5 hr of incubation and Northern blots were probed with, respectively, CP2 and D19 cDNAs. The low-intensity D19 mRNA band at \( 3 \times 10^{-2} \) M 8-CPT-cAMP is due to leakage during sample loading of the lane to the left.
derivative activates an inhibitory, rather than a stimulatory, pathway.

Activation of phospholipase C is mediated by at least one G protein. We measured whether 8-CPT-cAMP can increase GTP[yS] binding to membranes, which characterizes activation of G proteins (24, 45). Fig. 8 shows that cAMP induces a >2-fold increase of GTP[yS] binding. Half-maximal induction is achieved by 100 nM 8-Br-cAMP. 8-Br-cAMP induces the same increase as cAMP at 50-fold higher concentrations. 8-CPT-cAMP starts to increase GTP[yS] binding at the same concentrations as 8-Br-cAMP, but even at saturating concentrations, the 8-CPT-cAMP-induced increase is only half that induced by cAMP and 8-Br-cAMP. Apparently 8-CPT-cAMP cannot activate a subpopulation of G proteins.

**DISCUSSION**

We describe a cAMP derivative, 8-CPT-cAMP, which inhibits cAMP-induced chemotaxis at low concentrations, while inducing chemotaxis at supersaturating concentrations. 8-CPT-cAMP induces virtually normal accumulation of the second-messengers cAMP and cGMP but is defective in inositol phospholipid signaling and induces a decrease, rather than an increase, of InsP_3 levels. This effect of 8-CPT-cAMP can be explained by putative control of phospholipase C by both a stimulatory and an inhibitory G protein, with 8-CPT-cAMP only activating the inhibitory G protein. Compared with cAMP and 8-Br-cAMP, 8-CPT-cAMP shows a strongly reduced ability to increase the binding of GTP[yS] to Dictyostelium membranes (Fig. 8). This result indicates that 8-CPT-cAMP cannot activate a subpopulation of G proteins, presumably those responsible for phospholipase C activation.

The aberrant 8-CPT-cAMP-induced InsP_3 response may explain its behavior as a partial chemotactic antagonist. Studies using chemotactic mutants and introduction of second messengers into permeabilized cells have suggested that cGMP and InsP_3 signaling may respectively control myosin and actin polymerization (12, 17, 47, 48); 8-CPT-cAMP may antagonize chemotaxis by counteracting the cAMP-induced increase of InsP_3 levels. However, because 8-CPT-cAMP induces a normal cGMP response, this may, at saturating concentrations, suffice to induce some chemotaxis, perhaps due to enhanced cytokinesis.
The observation that 8-CPT-cAMP induces accumulation of cGMP but not of InsP₃ contradicts an earlier hypothesis that guanylate cyclase is activated by means of the InsP₃/Ca²⁺ pathway (49, 50). Remarkably, the cGMP response induced by 8-CPT-cAMP reaches much higher levels than that induced by cAMP (Fig. 5), which suggests that the cAMP-induced InsP₃ response may have a negative effect on cGMP accumulation. This hypothesis is supported by observations that both InsP₃ and Ca²⁺ strongly inhibit guanylate cyclase activity in vitro (51).

The ambiguous behavior of 8-CPT-cAMP on chemotaxis is also reflected in its effects on gene expression. 8-CPT-cAMP induces normal aggregative gene expression (Fig. 2) but is virtually ineffective in inducing postaggregative gene expression. 8-CPT-cAMP-induced gene expression may be mediated by cAMP, cGMP, InsP₃/Ca²⁺, or yet-unknown cAMP-induced responses. Earlier studies made involvement of cAMP in gene regulation unlikely because both aggregative and postaggregative gene expression occur under conditions that prevent adenylyl cyclase activation (5, 15, 16). FgdA mutants that are defective in the G protein, G₂, mediating phospholipase C activation (11, 13, 14), show no cAMP-induced expression of aggregative genes (16, 52) and no cAMP or cGMP responses (14, 53). It was suggested that G₂-mediated inositolphospholipid signaling mediates all cAMP-induced responses, including aggregative gene expression (11, 14, 53). The observation that 8-CPT-cAMP reduces InsP₃ levels but induces normal aggregative gene expression, as well as cAMP and cGMP accumulation, contradicts this suggestion. The defective G protein is possibly linked to other target proteins or could be required for an event early in development, which is required for subsequent differentiation.

Several data implicate InsP₃ in induction of prespore gene expression. Prespore gene expression cannot be induced by 8-CPT-cAMP, is effectively inhibited by Ca²⁺ antagonists (15, 54) and by LiCl which inhibits cAMP-induced InsP₃ accumulation (44), and can be induced under special conditions by InsP₃/diacylglycerol pulses (46). Expression of prestalk-related genes, such as CP2, is probably not mediated by InsP₃/Ca²⁺ because this response is not inhibited by Ca²⁺ antagonists (54) or lithium (44) and is counteracted by InsP₃/diacylglycerol pulses (46). Why 8-CPT-cAMP cannot induce CP2 gene expression is unclear. This response may be mediated by presently unknown intracellular messenger systems, which cannot be activated by 8-CPT-cAMP. The effects of 8-CPT-cAMP and lithium on the cGMP response correlate well with effects on aggregative gene expression. Both responses are effectively induced by 8-CPT-cAMP and stimulated by lithium (unpublished work), which suggest that cGMP may mediate induction of aggregative gene expression.

The present study shows that 8-CPT-cAMP is a very useful tool to unravel involvement of specific cAMP signal-transduction pathways in the great variety of cAMP-induced responses.

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