Molecular Biology of the Cell
Vol. 5, 703–711, June 1994

Two cAMP Receptors Activate Common Signaling Pathways in *Dictyostelium*

Robert H. Insall,* Ron D.M. Soede,† Pauline Schaap,† and Peter N. Devreotes*

*Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; and †Department of Biology, University of Leiden, 2333 AL Leiden, The Netherlands

Submitted December 18, 1993; Accepted May 12, 1994
Monitoring Editor: James A. Spudich

Multiple signal transduction pathways within a single cell may share common components. In particular, seven different transmembrane helix receptors may activate identical pathways by interacting with the same G-proteins. *Dictyostelium* cells respond to cAMP using one such receptor, cAR1, coupled by a typical heterotrimeric G-protein to intracellular effectors. However, cells in which the gene for cAR1 has been deleted are unexpectedly still able to respond to cAMP. This implies either that certain responses are mediated by a different receptor than cAR1, or alternatively that a second, partially redundant receptor shares some of the functions of cAR1.

We have examined the dose response and ligand specificity of one response, cAMP relay, and the dose response of another, cyclic GMP synthesis. In each case, the EC_{50} was ~100-fold higher and the maximal response was smaller in *car1*$^-$ than wild-type cells. These data indicate that cAR1 normally mediates responses to cAMP. The ligand specificity suggests that the responses seen in *car1*$^-$ mutants are mediated by a second receptor, cAR3.

To test this hypothesis, we constructed a cell line containing deletions of both cAR1 and cAR3 genes. As predicted, these lines are totally insensitive to cAMP. We conclude that the functions of the cAR1 and cAR3 receptors are partially redundant and that both interact with the same heterotrimeric G-protein to mediate these and other responses.

INTRODUCTION

The multicellular development of *Dictyostelium discoideum* is controlled by extracellular cAMP. During growth and feeding the cells live separately; when they starve, certain cells start periodically emitting cAMP, which attracts other starving cells. As well as moving, responding cells emit additional cAMP; this relay mechanism amplifies the signals and increases their range. By this means, aggregates of $\leq 10^5$ cells form, which then differentiate to form fruiting bodies. Several interrelated responses of cells to cAMP, including chemotaxis, cAMP relay, cyclic GMP (cGMP) synthesis, phospholipase C activation, and Ca++ influx have been extensively documented (reviewed in Devreotes, 1989). cAMP, acting either in intermittent waves or a constant concentration, also coordinates the developmental expression of several classes of genes (Darmon *et al.*, 1975; Kimmel, 1987).

The early effects of cAMP are transduced by cAR1, which is a member of the G-protein–linked family of receptors (Klein *et al.*, 1988). cAR1 expression, which is maximal while cells are aggregating, is strongly induced by cAMP waves, so cAMP signaling is reinforced by positive feedback. Three other cAMP receptors (cARs 2–4) have also been cloned (Saxe *et al.*, 1991a,b); all are expressed later in development. cAR3 levels rise toward the end of aggregation, whereas cARs 2 and 4 are maximally expressed in the slug and fruiting body stages (Saxe *et al.*, 1991a). Other components of the signal transduction pathway include the alpha and beta subunits (Gα2 and Gβ) of the principal G-protein coupled to cAR1 (Pupillo *et al.*, 1989; Lilly *et al.*, 1993) and the adenylyl cyclase (ACA) that effects cAMP relay (Pitt *et al.*, 1992). Cells in which the genes for cAR1, Gα2, Gβ, or ACA have been inactivated by homologous disruption are unable to develop or aggregate normally (Sun

et al., 1990; Sun and Devreotes, 1991; Pitt *et al.*, 1992; Lilly *et al.*, 1993). Both Gα2 and Gβ subunits are required for activation of second messengers in response to cAMP (Kumagai *et al.*, 1991; Lilly *et al.*, 1993). We have recently shown, however, that signaling in *car1*$^-$ cells can be restored by treatment with exogenous cAMP (Pupillo *et al.*, 1992). cAMP-stimulated *car1*$^-$ cells express several classes of cAMP-induced genes, including the aggregative marker csA and prestalk- and prespore-specific markers (Soede *et al.*, 1994). They can also activate adenylyl cyclase in vitro to a normal level, although its adaptation is impaired (Pupillo *et al.*, 1992).

In this work, we have examined how *car1*$^-$ cells respond to cAMP. We find that the dose responses of *car1*$^-$ cells to different agonists suggest that cAR1 mediates the activation of ACA and guanylyl cyclase in wild-type cells; in *car1*$^-$ cells, cAR3 is able to substitute partially for the functions of cAR1. We confirm this hypothesis by making a *car1*$^-$/*car3*$^-$ double gene disruptant and showing that both responses to extracellular cAMP are lost.

MATERIALS AND METHODS

General

Sp-cAMPS was obtained from Boehringer Mannheim (Indianapolis, IN). [2-^{3}H] Adenosine was purchased from Amersham International (Arlington Heights, IL). Unless explicitly stated, all other chemicals were obtained from Sigma (St. Louis, MO).

All *Dictyostelium* strains were grown in HL-5 medium (Watts and Ashworth, 1970), except during selection for or against the *pyr5-6* gene, when FM medium (Franke and Kessin, 1977) (from GIBCO BRL, Paisley, Scotland) was used. For development in suspension, cells were harvested, washed in DB (5 mM KH_2PO_4, 5 mM Na_2HPO_4, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$), resuspended at 2×10^7 cells/ml in DB, and shaken at 120 rpm with pulses of 300 nM cAMP every 6 min.

Perfusion Assay of cAMP Secretion

Perfusion was performed as described in Dinauer *et al.* (1980). To summarize, 10^7 *Dictyostelium* cells were shaken for 1–2 h with 1–3 100-μl aliquots of *Escherichia coli* labeled with tritiated adenosine (from Amersham International) according to the method of Devreotes *et al.* (1979). The cells were then washed once in DB, plated on DB/1% agar plates, and allowed to develop until waves were visible. For development in synergy, *car1*$^-$ or *car1*$^-$/*car3*$^-$ cells were plated along with 10^7 unlabeled AX3 cells. Cells were then washed off the plate in 1 ml DB and equally distributed among eight filters on a perfusion apparatus similar to that described in Dinauer *et al.* (1980). The filters were then perfused at a rate of 8–15 drops/min, with DB and with or without various concentrations of cAMP or Sp-cAMPS added. Fractions were collected every 20 s–1 min, and labeled cAMP was purified and measured according to Dinauer *et al.* (1980).

cAMP-induced cGMP Accumulation

Cells were harvested from axenic medium, washed, and stimulated with 300 nM cAMP pulses for 5 h at a density of 10^7 cells/ml in 10 mM phosphate buffer. Before the experiment, cells were concentrated to 2×10^8 cells/ml and aerated for 15 min. An aliquot of 270 μl cell suspension was stimulated with cAMP at t = 0, in the presence of 5 mM dithiothreitol. Reactions were terminated after 5, 10, 15, 30, and 60 s by transferring 30 μl cell suspension into 30 μl 3.5% perchloric acid (PCA). t = 0 samples were obtained by adding cAMP and PCA simultaneously. Samples were neutralized with 15 μl of a 50% saturated $KHCO_3$ solution and centrifuged for 2 mm at 8000 × *g*. The cGMP content of 25 μl of supernatant was assayed with a cGMP RIA kit (Amersham).

Generation of *car1*$^-$/*car3*$^-$ Cell Line

Transformation conditions were as described in Sun and Devreotes (1991), except that FM medium was used to select for URA+ transformants rather than HL5/G418.

The *car1*$^-$ cell line JS14 transformed with linearized pMYC10, a plasmid containing the pyr5-6 gene with most of the 5′ coding sequence deleted by removing a *Cla* I-*Pvu* II fragment. URA- clones were selected using HL5 containing 100 μg/ml 5-fluoroorotic acid (5-FOA), as described in Kalpaxis *et al.* (1990). One *car1*$^-$ URA- clone was designated RI-1; the deletion of the pyr5-6 gene was confirmed by genomic Southern blot as shown in Sun and Devreotes (1991). To make a *car1*$^-$ *car3*$^-$ mutant, RI-1 was transformed with the plasmid pRJ648, which contains two fragments of the cAR3 gene interspersed with a *Cla* I fragment containing the pyr5-6 gene. Transformants were selected for three weeks in FM medium, cloned on bacterial plates, and analysed by Southern blot. One *car1*$^-$ *car3*$^-$ line was picked and named RI-4; another, which contained a random insertion of pRJ648 and an intact cAR3 gene, was named RI-5.

Western Blotting

Cells were developed by shaking at 2×10^7 cells/ml in DB with 300 nM cAMP pulses every 6 min. Samples of 10^7 cells were taken every 2 h. Membranes were prepared by the method of Klein *et al.* (1988) and taken up in 100 μl sodium dodecyl sulfate sample buffer. Thirty microliters of dissolved membranes per sample were separated on a 7.5% acrylamide gel, electroblotted onto nitrocellulose, and probed with an ACA-specific antiserum (a kind gift of Dr. Carole Parent, P.N.D. lab). Antibody binding was visualised using enhanced chemiluminescence from Amersham.

RESULTS

cAMP Relay in *car1*$^-$ Cells

Pupillo *et al.* (1992) showed that *car1*$^-$ cells can respond to cAMP by activating ACA, as measured by an in vitro assay. To study cAMP-induced cAMP secretion (cAMP relay) in more detail, we labeled *car1*$^-$ cells with [^{3}H]-adenosine and measured labeled cAMP secretion under perfusion (Devreotes *et al.*, 1979; Devreotes and Steck, 1979; Dinauer *et al.*, 1980). Perfusion has several advantages over other methods of measuring relay. It measures the amount of cAMP secreted by intact cells. By using radiolabeled cells, it avoids confusion between secreted cAMP and the exogenous cAMP stimulus, and it enables the cAMP concentration to be clamped, irrespective of cAMP synthesis and breakdown by the cells. These advantages facilitate detailed studies of the kinetics and concentration dependence of cAMP relay.

When *car1*$^-$ cells that had been labeled with [^{3}H]-adenosine were mixed 1:1 with unlabeled wild-type cells and allowed to develop in synergy on nonnutrient agar, a relay response was clearly detected (Figure 1). The initial rise in the rate of cAMP secretion in *car1*$^-$ cells showed similar kinetics to that in wild-type cells, but the response subsided a little more rapidly. However,

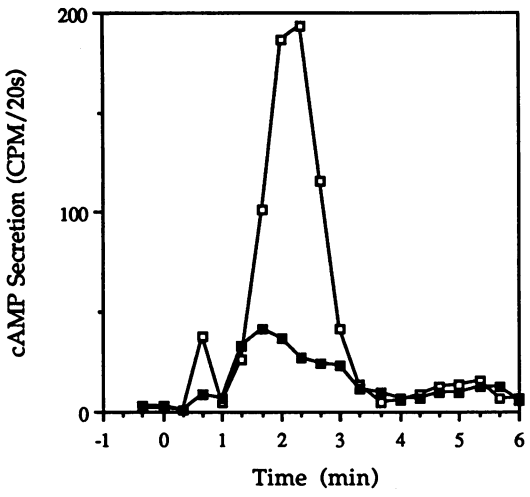


Figure 1. Perfusion assay of cAMP relay in wild-type and *car1*$^-$ cells. Wild-type cells (AX3, □) and *car1*$^-$ cells (JS14, ■) were labeled with [^{3}H]-adenosine, developed on agar, then washed onto filters, and perfused with DB. At time zero, the perfusing solution was changed to DB/100 μM cAMP. The amount of cAMP secreted by the cells was measured by scintillation counting after purifying cAMP from the eluate. The amounts of label remaining on the filters after perfusion were 4.08 × 10^6 cpm (AX3) and 3.06 × 10^6 cpm (JS14).

the peak secretion rate in *car1*$^-$ cells is only 20% of the wild-type rate. The unlabeled AX3 cells, which were added to enable the mutant cells to develop normally, did not affect the cAMP response of the labeled *car1*$^-$ cells, as shown below (for example in Figure 2).

To examine whether the same receptor mediates cAMP relay in *car1*$^-$ and wild-type cells, we measured the cAMP concentration dependence of both lines (Figure 2). As previously established, the response in wild-type cells was optimal at 100 nM cAMP (Figure 2a); above that concentration the size of the initial peak declined slightly. The response was biphasic. A second peak, about 7 min after stimulation, became more significant at high cAMP concentrations (Devreotes and Steck, 1979; Theibert *et al.*, 1986). In *car1*$^-$ cells, on the other hand, 100 nM cAMP barely elicited a response, and 30 μM was required to elicit a maximum response (Figure 2b). Also, the relay response in *car1*$^-$ cells was monophasic; the second peak was not detected, even at high cAMP concentrations. Figure 3, which shows the total cAMP secretion during the first and second peaks, summarizes these data. The half-maximal response of *car1*$^-$ cells requires a 10- to 100-fold higher concentration of cAMP than that in wild-type cells, depending on whether or not the second peak is included in the comparison.

These results suggest that cAR1 mediates both of the kinetic phases of the response of wild-type cells to a physiological stimulus. *car1*$^-$ cells require far higher concentrations of cAMP, and their maximal response is smaller.

cGMP Response in car1$^-$ *Cells*

cAMP-stimulated cGMP accumulation is controlled by a different biochemical pathway from that which mediates cAMP relay (Theibert and Devreotes, 1986) and is believed to be part of the mechanism of chemotaxis (Newell and Liu, 1992). Deletion of the cAR1 gene affected cGMP accumulation the same way it did cAMP relay; the response was again much smaller—30% of the wild-type response in the case of cGMP (Figure 4a)—but its timing was similar to the wild-type response. As with the cAMP relay response, the cGMP response required ~100-fold more cAMP for half-maximal stimulation in *car1*$^-$ than in wild-type cells (Figure 4b).

Differential Responses to a cAMP Analogue

The receptor that mediates these responses to cAMP in *car1*$^-$ cells could be a hitherto uncharacterized receptor or one of the other three members of the cAR family. There are no known genes >40–45% similar to cAR1 (Kimmel, personal communication). It is unlikely that

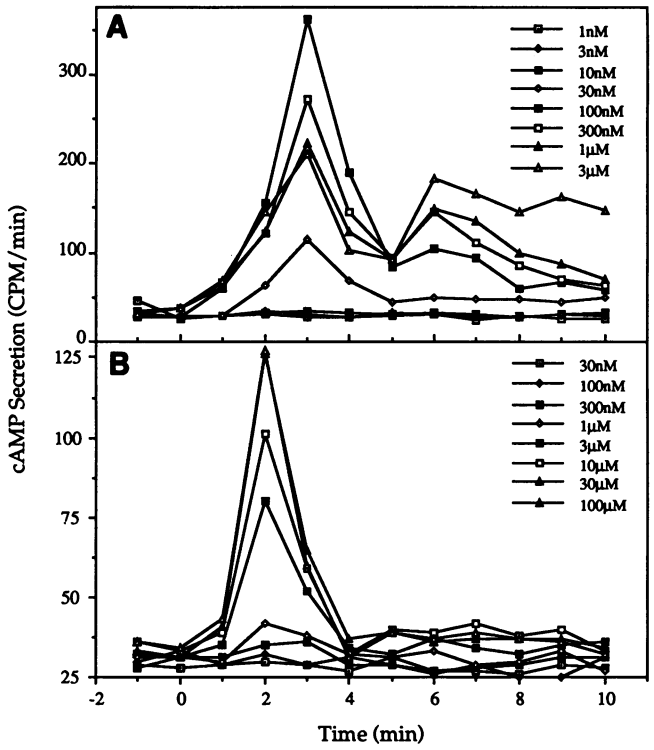


Figure 2. Relay response to different concentrations of cAMP in wild-type cells (AX3) in A and *car1*$^-$ cells (JS14) in B. Cells were labeled, developed, and perfused with DB as in Figure 1. To improve sensitivity, JS14 cells were labeled with twice as many bacteria as AX3. At time zero, the perfusing solution was changed to one containing the indicated cAMP concentration. Secreted cAMP was measured as in Figure 1. Total label per filter after perfusion was 1.42 × 10^6 cpm (AX3) and 2.63 × 10^6 (JS14).

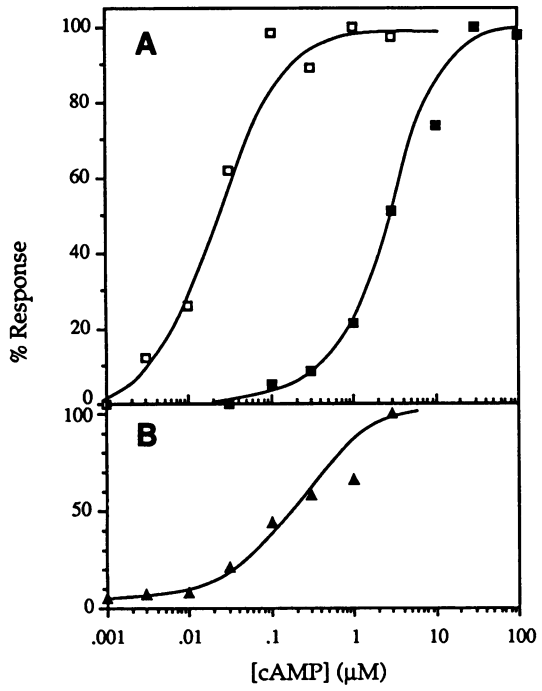


Figure 3. Dose response of cAMP relay in wild-type cells (AX3, □ and ▲) and *car1*$^-$ cells (JS14, ■). Cells were perfused with different concentrations of cAMP, and the total [^{3}H]-cAMP secretion during the first 4 min of stimulation (A) and the subsequent 6 min (B) was measured. Data show the mean from two separate experiments; each point is expressed as a percentage of peak secretion. After correction for the intensity of labeling, secretion after a saturating stimulus was 5.7× greater in wild-type than JS14 cells.

the responses are mediated by cARs 2 and 4, because they are specifically expressed in prestalk cells (Saxe *et al.*, 1993) and are first detected late in development. cAR3, which is the most likely alternative to cAR1, is not easily detectable by Northern blot before 6 h (Saxe *et al.*, 1991a); the number of cAR3 molecules is very low at the developmental stage used in these assays (Soede *et al.*, 1994).

To assess whether cAR3 mediates responses to cAMP in the absence of cAR1, we repeated the dose-response experiment using the cAMP analogue Sp-cAMPS. Sp-cAMPS binds to cAR1 with >100-fold lower affinity than cAMP; in contrast, it binds to cAR3 with only five-fold lower affinity (Johnson *et al.*, 1992). As illustrated in Figure 5, ~100-fold more Sp-cAMPS than cAMP was required to elicit an equivalent response in wild-type cells. For *car1*$^-$ cells, the difference was only about fivefold. This observation suggests that cAR3 is the receptor that mediates responses to cAMP in *car1*$^-$ cells.

Construction of a car1$^-$/car3$^-$ Cell Line

To further assess whether cAR3 substitutes for cAR1 in *car1*$^-$ cells, we constructed a cAR1 and cAR3 double gene disruptant. The duplicated gene encoding cAR1 in AX3 were originally deleted by homologous recombination using a G418 resistance marker to produce strain JS14 (Sun and Devreotes, 1991). We then deleted the *pyr5-6* gene in JS14 using a construct containing an internal deletion (pMYC10, a generous gift from Mei-Yu Chen, P.N.D. lab), followed by selection of *pyr5-6*$^-$ clones in 5-FOA (Boy-Marcotte *et al.*, 1984; Kalpaxis *et al.*, 1990), to give a *car1*$^-$ *pyr5-6*$^-$ strain, RI-1 (Figure 6a). The cAR3 gene in RI-1 was then disrupted by homologous recombination using a *pyr5-6*$^+$ marker and selection for uracil prototrophy, to give strains RI-4 (*car1*$^-$ *car3*$^-$) and RI-5 (*car1*$^-$ *car3*$^+$), as shown in Figure 6b. RI-5, in which *pyr5-6* incorporated by random insertion rather than homologous recombination, serves as a control for the effects of transformation and selection; it behaves identically to its original parent, JS14, in both development and cAMP signaling (unpublished observations). Both cell lines appear normal in size and appearance and grow with a doubling time of 10–11 h, identical to wild-type AX3 cells.

Signaling in car1$^-$/car3$^-$ Cells

We measured cAMP relay under the same conditions as in Figure 1, using [^{3}H]-labeled *car1*$^-$/*car3*$^-$ cells de-

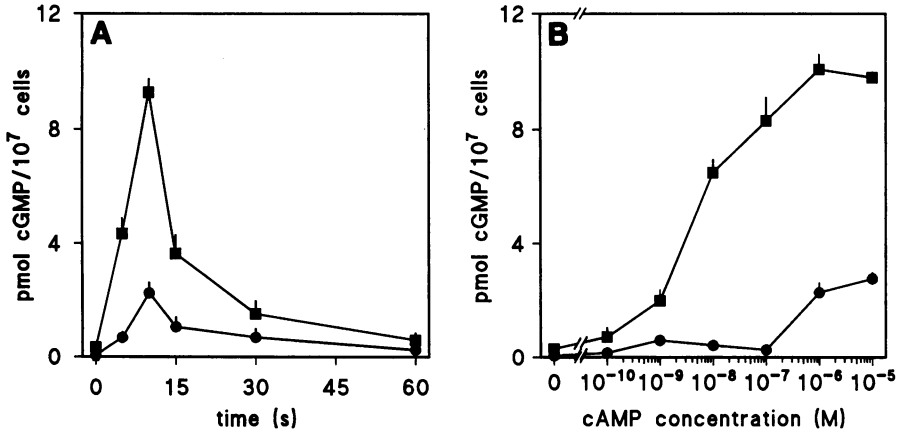


Figure 4. cGMP response in wild-type and *car1*$^-$ cells. (A) cGMP response in AX3 (■) and *car1*$^-$ (●) cells after stimulation with 1 μM cAMP. (B) Dose-response after 10 s of stimulation with the indicated cAMP concentrations in AX3 (■) and *car1*$^-$ (●) cells. Means and SEM of three experiments performed in triplicate are presented.

veloped in synergy with AX3. Even when these cells were perfused with 100 μM cAMP, no relay response was discernible (Figure 7a). An in vitro assay of cAMP stimulation of ACA activity gave similar results; the ACA activation seen in *car1*$^-$ cells was essentially lost in the *car1*$^-$/*car3*$^-$ cells (Pupillo *et al.*, 1992; unpublished observations). Likewise, after *car1*$^-$/*car3*$^-$ cells had been pulsed with cAMP for 5 h, they exhibited no detectable cAMP-induced cGMP synthesis (Figure 7b). The additional loss of cAR3 leads to the complete loss of even the weakened responses observed in *car1*$^-$ cells, although the disruption of the cAR3 gene in the presence of cAR1 has no visible effect on development or cAR1 expression (Johnson *et al.*, 1992; Devreotes, unpublished data). Thus in the absence of cAR1, cAR3 is required for signaling in response to cAMP.

G-protein and Adenylyl Cyclase Levels in the car1$^-$*/car3*$^-$ *Double Mutant*

One possible reason for the absence of agonist-stimulated cAMP and cGMP synthesis in *car1*$^-$ cells could be poor expression of developmentally controlled proteins involved in the cAMP signaling pathways. Because cAMP signaling induces expression of many of the components of the signaling pathways themselves, receptor mutants might have decreased quantities of these effectors. This would diminish cAMP and cGMP synthesis even if cells could detect external cAMP signals. We therefore used western blots to examine the levels of Gα2 and ACA in cAMP-pulsed wild-type and *car1*$^-$/*car3*$^-$ cells (Figure 8a). Gα2 levels are similar in wild-type and mutant cells; ACA, however, is expressed at a diminished level in *car1*$^-$/*car3*$^-$ cells.

One possible explanation for the lack of cAMP-induced cAMP secretion in *car1*$^-$/*car3*$^-$ cells is that the amount of ACA protein is too small to generate a measurable response. To rule out this possibility, we measured ACA activity in vitro after stimulation with GTPγS, which directly activates ACA through G-proteins, bypassing the need for receptors (Figure 8b). Even though *car1*$^-$/*car3*$^-$ cells contain less ACA, their GTPγS-stimulated ACA activity is similar to that in wild-type cells. The lack of a cAMP relay response in *car1*$^-$/*car3*$^-$ cells is therefore apparently because of the absence of surface cAMP receptors; mutant cells should be able to generate a measurable response if they could perceive a cAMP stimulus.

DISCUSSION

Two Receptors Share a Common G-protein

When the gene for Gα2 is deleted, many cAMP induced functions are lost, including gene expression, chemotaxis, cAMP relay, and cGMP synthesis (Kumagai *et al.*, 1991). No other Gα-protein deletion has significant effects on any of these responses (Wu *et al.*, 1994). We have shown here that deletion of the cAR1 gene leads to a drop in the sensitivity of each response, and deletion of both cAR1 and cAR3 leads to the loss of both. It therefore seems most likely that cAR1 and a second receptor, which appears to be cAR3, both couple to Gα2.

The other two cARs, 2 and 4, are very similar in sequence to cARs 1 and 3 (Saxe *et al.*, 1991a), so it may be that they also couple to Gα2. This is harder to investigate, as their functions both lie late in development.

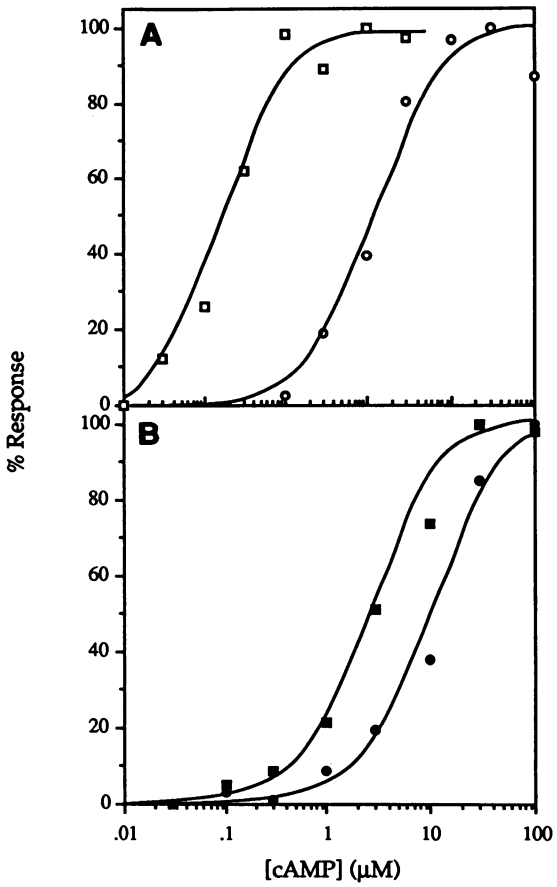


Figure 5. Dose responses of cAMP relay to cAMP and Sp-cAMPS. (A) Response of wild-type (AX3) cells to different concentrations of Sp-cAMPS (○) and cAMP (□) (data replotted from Figure 3). (B) Response of *car1*$^-$ (JS14) cells to different concentrations of Sp-cAMPS (●) and cAMP (■) (data replotted from Figure 3). Each point shows the mean from two separate experiments; data are expressed as a percentage of peak secretion.

Diminished Sensitivity and Response Levels in car1$^-$ *Cells*

The 10- to 100-fold difference in sensitivity to cAMP between wild-type and *car1*$^-$ cells is greater than would be predicted from the differences in binding affinities between cAR1 and cAR3. cAR1 has K_ds of 25 (5% of sites) and 230 nM (95% of sites) for cAMP (measured under physiological conditions), whereas cAR3 has a

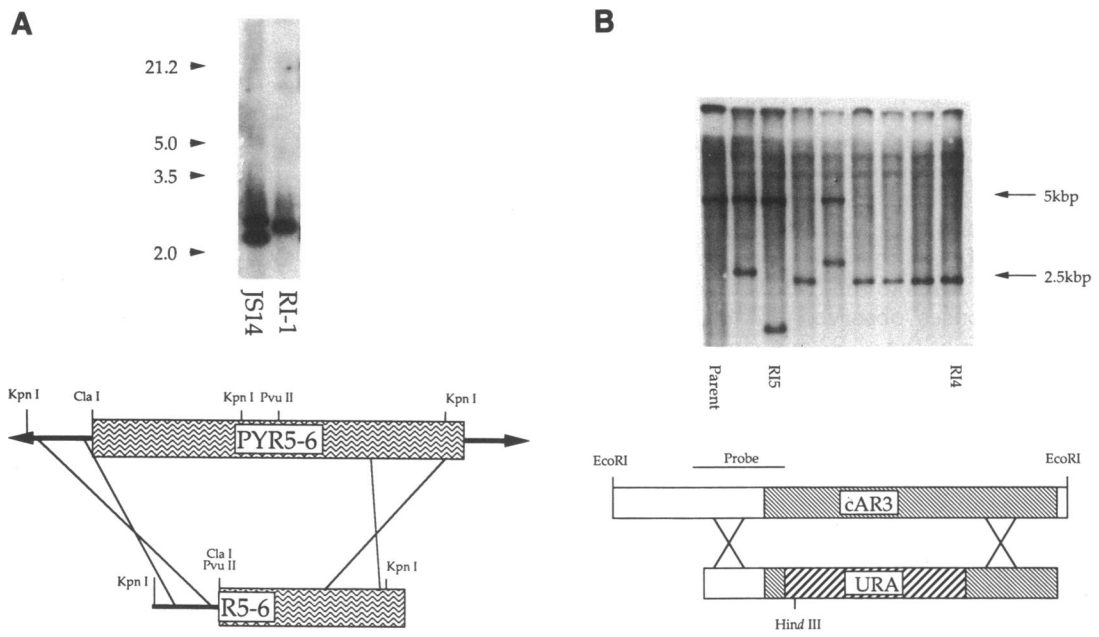


Figure 6. Construction of strains. (A) Construction of *car1*$^-$ URA- strain (RI-1) from *car1*$^-$ (JS14). JS14 cells were transformed with pMYC10, a vector containing a deletion of the 5′ end of the pyr5-6 gene, and homologous recombinants were selected using 5-FOA. The deletion was confirmed by Southern blot; genomic DNA was cut with *Kpn* I, separated on a 1% agarose gel, blotted onto nitrocellulose, and probed with a complete cloned pyr5-6 fragment. The left lane (JS14) shows two bands, the right lane (RI-1) only one. (B) Construction of *car1*$^-$/*car3*$^-$ strain (RI-4) from *car1*$^-$ URA- strain (RI-1). RI-1 cells were transformed with a vector containing the cAR3 cDNA with the central portion replaced with a URA gene, and homologous recombinants were selected by growth in FM medium. The deletion was confirmed by Southern blot; genomic DNA was cut with *Eco*RI and *Hin*dIII, separated on a 0.8% agarose gel, blotted onto nitrocellulose, and probed with the indicated cAR3 fragment. The left lane (RI-1, the parent) shows a single 5-kilobase (kb) band; in cAR3-deleted strains (lanes 4, 6, 7, 8 and 9; RI-4 is lane 9) the 5-kb cAR3 band is replaced by the 2.5-kb construct. Other strains (lanes 2, 3 and 5; lane 3 is RI-5) contain an unaltered cAR3 gene and a random integration of the construct.

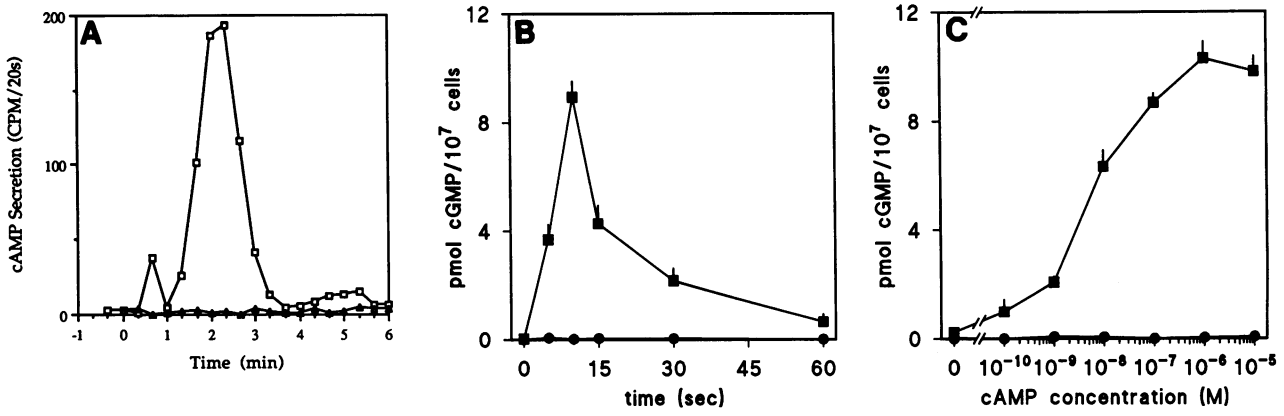
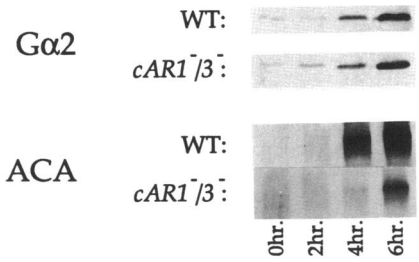


Figure 7. Complete loss of signaling in *car1*$^-$/*car3*$^-$ cells. (A) cAMP relay. *car1*$^-$/*car3*$^-$ cells (RI-4, △) and wild-type cells (AX3, □) were labeled, developed, and perfused as in Figure 1. At time zero, the perfusing solution was changed to DB/100 μM cAMP. The amount of cAMP secreted by the cells was measured by scintillation counting after purifying cAMP from the eluate as described in the text. The AX3 data are also plotted in Figure 1. The amounts of label remaining on the filters after perfusion were 4.08×10^6 cpm (AX3) and 2.09×10^6 cpm (RI4). (B) cGMP response in AX3 (■) and *car1*$^-$/*car3*$^-$ (●) cells after stimulation with 1 μM cAMP. (C) Dose response of cGMP synthesis after 10 s of stimulation with the indicated cAMP concentrations in AX3 (■) and *car1*$^-$/*car3*$^-$ (●) cells. Means and SEM of three experiments performed in triplicate are presented.

A



B

Cell Type:	GTPγS:	ACA Activity (pmol/min/mg):
WT	-	0.39
	+	21.2
cAR1⁻/3⁻	-	2.26
	+	18.8

Figure 8. (A) Gα2 and ACA expression in wild-type (AX3) and *car1⁻/car3⁻* (RI-4) strains. Cells were shaken in DB at 2×10^7 cells/ml with 300 nM cAMP pulses every 6 min. Samples were taken every 2 h, and membrane fractions were prepared. Membranes from 3×10^6 cells at each point were separated on a 7.5% acrylamide gel, blotted, and probed with Gα2- and ACA-specific antisera. (B) Stimulation of ACA by GTPγS. AX3 and RI-4 cells were shaken in DB at 2×10^7 cells/ml with 300 nM cAMP pulses every 6 min for 6 h, then lysed in the presence or absence of GTPγS and assayed for ACA activity exactly as described in Pupillo *et al.* (1992).

K_ds of 47 and 680 nM (Johnson *et al.*, 1992). This might at first appear to be inconsistent with the hypothesis that cAR3 replaces cAR1 function. One possible explanation is that the actual concentration of cAMP required for a half-maximal response in wild-type cells is lower (EC_{50} = 10 nM) than would be expected from the K_d of the majority of the sites (van Haastert, 1985; Theibert *et al.*, 1986). It therefore seems that some other component of the cAMP response pathway is saturated at a low occupancy of cAR1. Because cAR3 is expressed at a far lower level than cAR1 during normal development and at a lower level still in *car1⁻* cells (Soede *et al.*, 1994), a higher proportion of those cAR3 molecules must be occupied by ligand to generate a similar response.

The small quantity of cAR3 might also explain the diminished magnitude of the cAMP relay and cGMP responses. Because development is generally less efficient in *car1⁻* cells, even after treatment with cAMP pulses, and many of the components of the signal transduction pathways are controlled by positive feedback, the diminished responses seen in Figures 2 and 4 are unsurprising. The smaller cAMP response we observed in *car1⁻* cells appears to disagree with the results described in Pupillo *et al.* (1992), which showed relay peaking at a similar level in wild-type and *car1⁻* cells. However, the ACA activation described in Pupillo *et al.* (1992) was measured in cell lysates. We presume that the in vivo assays described here are limited by the amount of ACA, whereas the activity measured by the in vitro assay is limited by some other factor. One candidate is CRAC, a cytoplasmic protein needed for ACA activation, that is inevitably considerably diluted in cell lysates (Lilly and Devreotes, 1994).

Adaptation in car1⁻ Cells

Our data show that the kinetics of both the cGMP and cAMP relay responses in *car1⁻* cells are not significantly different from those in wild-type cells. This observation contrasts those of Pupillo *et al.* (1992), who found that adaptation of cAMP relay in *car1⁻* cells was considerably slower than wild-type. Again, this difference is probably caused by the different assays. Pupillo *et al.* (1992) only assayed the activation of ACA, whereas we measure secretion of cAMP from whole cells. The drop in cAMP secretion during persistent cAMP stimulation could be caused by several factors, including a rise in intracellular phosphodiesterase activity and phosphorylation of the receptor and G-protein. Pupillo *et al.* (1992) also measured adaptation as the inability of GTPγS to activate ACA in lysed cells and showed that this occurs by a positive inactivation of ACA, which is independent of Gα2 (Snaar-Jagalska and van Haastert, 1990). In our in vivo assays, the cAR1-mediated inactivation of ACA may only play a minor role.

Soede *et al.* (1994) also show a lack of adaptation in *car1⁻* cells. Continuous stimulation with cAMP normally inhibits cAMP-induced gene expression (Schaap and van Driel, 1985), presumably by adaptating the signal transduction system. In *car1⁻* cells, however, continuous cAMP increases the levels of several cAMP-controlled mRNAs (Soede *et al.*, 1994). Thus the inhibition of gene expression, like the inactivation of ACA, seems to be poorly mediated by cAR3.

Partial Redundancy of two cAMP Receptors

The work described here shows that cells in which the gene for cAR1 has been deleted remain responsive to external cAMP signals. Compared to wild-type cells, however, the magnitudes of the responses are smaller and the amount of cAMP needed to elicit them is increased. These results strongly suggest that cAR1 mediates cAMP signaling during normal development, but in its absence another receptor is able to substitute. Two lines of evidence suggest that the second receptor is cAR3. First, the cAMP analogue Sp-cAMPS is >100-fold less potent than cAMP at eliciting a cAMP relay response in wild-type cells but only fivefold less potent in *car1⁻* cells; this corresponds to a 110-fold and fivefold difference in the binding affinities of these agonists to cAR1 and cAR3 respectively. Second, if the gene for

cAR3 is deleted from *car1*$^-$ cells, all responses to cAMP are lost.

It remains possible that either cAR1 or cAR3 is required for expression of some third protein, and lack of this protein prevents signaling in *car1*$^-$/*car3*$^-$ cells. Parallel work shows that *car1*$^-$/*car3*$^-$ cells have no cAMP-induced gene expression (Soede *et al.*, 1994). However, considering all the data as a whole it seems most likely that cAR3 is responsible for cAMP and cGMP production as well as gene expression in *car1*$^-$ cells. In *car1*$^-$ cells developed alone on nonnutrient agar, some unexpected chemotactic sensitivity appears after about 13 h (Sun and Devreotes, 1991). This may now be explained as late expression of cAR3, delayed by the absence of exogenous cAMP pulses. Chemotaxis in the cAR1 cells required more than 10-fold higher cAMP concentrations, which is also consistent with a role for cAR3.

The apparent partial redundancy between cAR1 and cAR3 also helps to explain why *car3*$^-$ cells can develop normally. In *car3*$^-$ cells, cAR1 is presumably able to make up for the functions of cAR3. We presume that cAR3 appeared earlier in evolution than cAR1; *Dictyostelium* species that use other chemoattractants during aggregation still use cAMP late in development (Schaap *et al.*, 1984) at a time when cAR3 would normally be expressed. It will be interesting to examine the different cAR genes present in these species. In view of the lack of any discernible phenotype of *car3*$^-$ cells, it might seem strange that the cAR3 gene has not been lost from *D. discoideum*. We have speculated that their development is affected in some way that is not detected under standard laboratory conditions (Johnson *et al.*, 1992).

ACKNOWLEDGMENTS

We are grateful to Dr. Carole Parent for the ACA antibody, Mei-Yu Chen for pMYC10, and to Mike Caterina and Dr. Carole Parent for helpful comments on the text. This work was supported by National Institutes of Health grant GM-34933 to P.N.D., GB-MW grant 900-546-077 from the Netherlands Organization for Scientific Research, and a Science and Engineering Research Council North Atlantic Treaty Organization fellowship to R.H.I.

REFERENCES

Boy-Marcotte, E., Vilaine, F., Camonis, J., and Jacquet, M. (1984). A DNA sequence from *Dictyostelium discoideum* complements the URA3 and URA5 mutations of *S. cerevisiae*. Mol. Gen. Genet. *193*, 406–413.

Darmon, M., Brachet, P., and Pereira da Silva, L.H. (1975). Chemotactic signals induce cell differentiation in *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. *72*, 3163–3166.

Devreotes, P.N. (1989). *Dictyostelium discoideum:* a model system for cell-cell interactions in development. Science *245*, 1054–1058.

Devreotes, P.N., Derstine, P.L., and Steck, T.L. (1979). Cyclic 3′,5′-AMP relay in *Dictyostelium discoideum*. I. A technique to monitor responses to controlled stimuli. J. Cell Biol. *80*, 291–299.

Devreotes, P.N., and Steck, T.L. (1979). Cyclic 3′,5′-AMP relay in *Dictyostelium discoideum*. II. Requirements for the initiation and termination of the response. J. Cell Biol. *80*, 300–309.

Dinauer, M.C., MacKay, S.A., and Devreotes, P.N. (1980). Cyclic 3′,5′-AMP relay in *Dictyostelium discoideum*. III. The relationship of cAMP synthesis and secretion during the cAMP signaling response. J. Cell Biol. *86*, 537–544.

Franke, J., and Kessin, R. (1977). A defined minimal medium for axenic strains of *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. **74**, 2157–2161.

Johnson, R., van Haastert, P.J.M., Kimmel, A., Saxe, C., Jastorff, B., and Devreotes, P.N. (1992). The cyclic nucleotide specificity of three cAMP receptors in *Dictyostelium*. J. Biol. Chem. *267*, 4600–4607.

Kalpaxis, D., Werner, H., Marcotte, E.B., Jacquet, M., and Dingermann, T. (1990). Positive selection for *Dictyostelium* mutants lacking uridine monophosphate synthase activity based on resistance to 5-fluoro-orotic acid. Dev. Genet. *11*, 396–402.

Kimmel, A.R. (1987). Different molecular mechanisms for cAMP regulation of gene expression during *Dictyostelium* development. Dev. Biol. *122*, 163–171.

Klein, P.S., Sun, T.J., III, Saxe, C.L., Kimmel, A.R., Johnson, R.L., and Devreotes, P.N. (1988). A chemoattractant receptor controls development in *Dictyostelium*. Science *241*, 1467–1472.

Kumagai, A., Hadwiger, J.A., Pupillo, M., and Firtel, R.A. (1991). Molecular genetic analysis of two Gα protein subunits in *Dictyostelium*. J. Biol. Chem. *266*, 1220–1228.

Lilly, P.J., and Devreotes, P.N. (1994). Purification and identification of a cytosolic regulator required for guanine nucleotide stimulation of adenylyl cyclase in *Dictyostelium*. J. Biol. Chem. **269**, 14123–14129.

Lilly, P.J., Wu, L., Welker, D.L., and Devreotes, P.N. (1993). A G-protein β subunit is essential for *Dictyostelium* development. Genes & Dev. *7*, 986–995.

Newell, P.C., and Liu, G. (1992). Streamer F mutants and chemotaxis of *Dictyostelium*. Bioessays *14*, 473–479.

Pitt, G.S., Milona, N., Borleis, J., Lin, K.C., Reed, R.R., and Devreotes, P.N. (1992). Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. Cell *69*, 305–315.

Pupillo, M., Insall, R.H., Pitt, G.S., and Devreotes, P.N. (1992). Multiple cyclic AMP receptors are linked to adenylyl cyclase in *Dictyostelium*. Mol. Biol. Cell *3*, 1229–1234.

Pupillo, M., Kumagai, A., Pitt, G.S., Firtel, R.A., and Devreotes, P.N. (1989). Multiple alpha subunits of guanine nucleotide-binding proteins in *Dictyostelium*. Proc. Natl. Acad. Sci. USA *86*, 4892–4896.

Saxe, C.L., Ginsburg, G.T., Louis, J.M., Johnson, R., Devreotes, P.N., and Kimmel, A.R. (1993). CAR2, a prestalk cAMP receptor required for normal tip formation and late development of *Dictyostelium discoideum*. Genes & Dev. *7*, 262–272.

Saxe, C.L., Johnson, R., Devreotes, P.N., and Kimmel, A.R. (1991a). Multiple genes for cell surface cAMP receptors in *Dictyostelium discoideum*. Dev. Genet. *12*, 6–13.

Saxe, C.L., Johnson, R.L., Devreotes, P.N., and Kimmel, A.R. (1991b). Expression of a cAMP receptor gene of *Dictyostelium* and evidence for a multigene family. Genes & Dev. *5*, 1–8.

Schaap, P., Konijn, T.M., and van Haastert, P.J.M. (1984). cAMP pulses coordinate morphogenetic movement during fruiting body formation of *Dictyostelium minutum*. Proc. Natl. Acad. Sci. USA *81*, 2122–2126.

Schaap, P., and van Driel, R. (1985). Induction of post-aggregative differentiation in *Dictyostelium discoideum* by cAMP: evidence for the involvement of the cell surface cAMP receptor. Exp. Cell Res. *159*, 388–398.

Snaar-Jagalska, B.E., and van Haastert, P.J.M. (1990). Pertussis toxin inhibits cAMP-induced desensitization of adenylate cyclase in *Dictyostelium discoideum*. Mol. Cell. Biochem. *92*, 177–189.

Soede, R.D.M., Insall, R.H., Devreotes, P.N., and Schaap, P. (1994). Extracellular cAMP can restore development in *Dictyostelium* cells lacking one, but not two, subtypes of early cAMP receptors. Development (*in press*).

Sun, T.J., and Devreotes, P.N. (1991). Gene targeting of the aggregation stage cAMP receptor cAR1 in *Dictyostelium*. Genes & Dev. *5*, 572–582.

Sun, T.J., van Haastert, P.J.M., and Devreotes, P.N. (1990). Surface cAMP receptors mediate multiple responses during development in *Dictyostelium*—evidenced by antisense mutagenesis. J. Cell Biol. *110*, 1549–1554.

Theibert, A., and Devreotes, P.N. (1986). Surface receptor-mediated activation of adenylate cyclase in *Dictyostelium:* regulation by guanine nucleotides in wild-type cells and aggregation deficient mutants. J. Biol. Chem. *261*, 15121–15125.

Theibert, A., Palmisano, M., Jastorff, B., and Devreotes, P. (1986). The specificity of the cAMP receptor mediating activation of adenylate cyclase in *Dictyostelium discoideum*. Dev. Biol. *114*, 529–533.

van Haastert, P.J.M. (1985). The modulation of cell surface cAMP receptors from *Dictyostelium discoideum* by ammonium sulfate. Biochim. Biophys. Acta *845*, 254–260.

Watts, D., and Ashworth, J. (1970). Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. Biochem. J. *119*, 171–174.

Wu, L., Gaskins, C., Zhou, K., Firtel, R.A., and Devreotes, P.N. (1994). Cloning and targeted mutations of $G\alpha 7$ and $G\alpha 8$, two developmentally regulated G protein α-subunit genes in *Dictyostelium*. Mol. Biol. Cell *5*, 691–702.