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The Surface Cyclic AMP Receptors, cAR1, cAR2, and cAR3, Promote Ca^{2+} Influx in *Dictyostelium discoideum* by a G_α2-Independent Mechanism

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Activation of surface folate receptors or cyclic AMP (cAMP) receptor (cAR) 1 in *Dictyostelium* triggers within 5–10 s an influx of extracellular Ca^{2+} that continues for 20 s. To further characterize the receptor-mediated Ca^{2+} entry, we analyzed $^{45}Ca^{2+}$ uptake in amoebas overexpressing cAR2 or cAR3, cARs present during multicellular development. Both receptors induced a cAMP-dependent Ca^{2+} uptake that had comparable kinetics, ion selectivity, and inhibitor profiles as folate- and cAR1-mediated Ca^{2+} uptake. Analysis of mutants indicated that receptor-induced Ca^{2+} entry does not require G protein α subunits $G_\alpha1$, $G_\alpha2$, $G_\alpha3$, $G_\alpha4$, $G_\alpha7$, or $G_\alpha8$. Overexpression of cAR1 or cAR3 in $g_\alpha2^-$ cells did not restore certain $G_\alpha2$-dependent events, such as aggregation, or cAMP-mediated activation of adenylate and guanylate cyclases, but these strains displayed a cAMP-mediated Ca^{2+} influx with kinetics comparable to wild-type aggregation-competent cells. These results suggest that a plasma membrane-associated Ca^{2+}-influx system may be activated by at least four distinct chemoreceptors during *Dictyostelium* development and that the response may be independent of G proteins.

INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* is amenable for biochemical and genetic studies on the role of transmembrane signaling pathways in growth and development. Growing amoebas of this organism live as single cells and respond chemotactically to folate, a compound secreted by the bacterial food source (Pan *et al.*, 1972). After nutrient exhaustion, cells develop the ability to periodically synthesize and secrete cyclic AMP (cAMP).[1] These oscillations of extracellular cAMP are relayed to more distal cells, and the resulting chemical waves cause amoebas to move toward the aggregation center. Approximately 30 cAMP oscillations are required to give rise to a multicellular aggregate of $\sim 10^5$ cells, which then undergoes a program of events involving differentiation and morphogenesis, resulting in the formation of a fruiting body (reviewed in Devreotes, 1982).

[1] Abbreviations used: cAMP, cyclic AMP; cAR, cAMP receptor; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; *Fgd A*, *Frigid A*; cGMP, cyclic GMP.

The folate- and cAMP-mediated chemosensory pathways of *Dictyostelium* appear to be analogous to the transmembrane signaling pathways of higher eukaryotic cells. A cAMP stimulus elicits a fast increase in inositol 1,4,5-trisphosphate (Europe-Finner and Newell, 1987; Van Haastert *et al.*, 1989) and a slower increase in intracellular cAMP (Wurster *et al.*, 1977), resulting from activation of phospholipase C (Europe-Finner *et al.*, 1989) and adenylate cyclase activities (Roos and Gerisch, 1976), respectively. Folate and cAMP also trigger several common events, including a transient elevation of intracellular cyclic GMP (cGMP) (Mato *et al.*, 1977b) and an influx of extracellular Ca^{2+} (Wick *et al.*, 1978; Bumann *et al.*, 1984; Milne and Coukell, 1991). Biochemical evidence suggests that both chemoreceptors couple to effector enzymes through intermediary guanine nucleotide-binding proteins (G proteins) (Janssens and Van Haastert, 1987; Van Haastert and Devreotes, 1993). The major class of cAMP binding sites of aggregating cells has been cloned. This cAMP receptor (cAR) 1 possesses seven transmembrane-spanning domains, a topology comparable with that of mammalian G protein-coupled receptors such as rhodopsin (Klein *et al.*,

1988). More recently, genes encoding three related cAMP receptors, which are homologous to cAR1 and expressed during multicellular development (cAR2, cAR3, and cAR4), have been isolated (Saxe *et al.*, 1991, 1992; Johnson *et al.*, 1992a; Louis, Ginsburg, and Kimmel, personal communication). Eight distinct G protein α subunits (Pupillo *et al.*, 1989; Hadwiger *et al.*, 1991; Wu and Devreotes, 1991; Pupillo and Devreotes, unpublished data) and a single G protein β subunit (Pupillo *et al.*, 1988), which display considerable homology with their respective mammalian counterparts, also have been cloned. Taken together, these findings strongly implicate the importance of G protein-mediated signaling pathways during *Dictyostelium* development.

The clearest example of receptor/G protein/effector coupling in *Dictyostelium* derives from analysis of mutants of the complementation group *Frigid A* (*Fgd A*) (Coukell *et al.*, 1983), which are defective in the G protein α subunit $G_\alpha 2$ (Firtel *et al.*, 1989). Vegetative *Fgd A* cells exhibit normal folate-induced signaling events and respond chemotactically to folate (Kesbeke *et al.*, 1990). Developing amoebas express cAMP binding sites (Kesbeke *et al.*, 1988) but fail to undergo chemotaxis (Coukell *et al.*, 1983) or to synthesize cAMP, cGMP (Kesbeke *et al.*, 1988), or inositol 1,4,5-trisphosphate (Snaar-Jagalska *et al.*, 1988) in response to exogenous cAMP. These findings have led to the proposal that cAR1 activates certain effectors through $G_\alpha 2$. On the other hand, when *Fgd A* mutants were repeatedly stimulated with cAMP, they eventually exhibited low but reproducible levels of cAMP-stimulated Ca^{2+} entry (Milne and Coukell, 1991), suggesting that certain cAR-mediated events do not require $G_\alpha 2$. In this study, we test whether cAR1 triggers separate $G_\alpha 2$-dependent and $G_\alpha 2$-independent signaling pathways by characterizing cAMP-activated Ca^{2+} influx in $g_\alpha 2^-$ (*Fgd A*) cells overexpressing cAMP receptors. In addition, we have assessed whether other G protein α subunits, or cARs other than cAR1, regulate Ca^{2+} influx in this organism. Our results suggest that cAR1, cAR2, cAR3, and the folate receptor promote Ca^{2+} entry through a pathway that is independent of each of the examined G protein α subunits.

MATERIALS AND METHODS

Materials

Materials used and their sources were as follows: cGMP [^{125}I] scintillation proximity assay system (Amersham, Arlington Heights, IL); $^{45}CaCl_2$ (8.0–9.9 mCi/mg) and [^{125}I]Protein A (8.7 μCi/μg) (ICN Biomedicals, Irvine, CA); [^{3}H]cAMP, ammonium salt (31.4 Ci/mmol)(New England Nuclear, Boston, MA); $CoCl_2 \cdot 6H_2O$, $CdCl_2$, folate, and Ponceau S concentrate (Sigma Chemical, St. Louis, MO); nitrocellulose, pore size 0.45 μm (Schleicher & Schuell, Keene, NH). Other materials were of analytical grade and purchased from the suppliers indicated in Milne and Coukell (1991).

Strains and Culture Conditions

The following *Dictyostelium* strains were used in this study: AX3 (Williams *et al.*, 1974) and AX3 transformed with plasmid pBS18B6 containing the cAR1 gene or transformed with a control vector lacking receptor sequence (Klein *et al.*, 1988); Δ208 cells, a cAR1$^-$ mutant (Sun and Devreotes, 1991); Δ208 cells transformed with expression constructs containing cAR1, cAR2, or cAR3 (cAR1/Δ208, cAR2/Δ208, and cAR3/Δ208 cells, respectively) (Johnson *et al.*, 1991, 1992b). Henceforth, Δ208 cells and Δ208 cells overexpressing cAR1, cAR2, or cAR3 will be called cAR1$^-$, cAR1/cAR1$^-$, cAR2/cAR1$^-$, and cAR3/cAR1$^-$, respectively. Null mutants of different G protein α subunits were also used: MP2, HPS400-derived $g_\alpha 2^-$ cells (provided by M. Pupillo, Wayne State University, Detroit, MI); MP3, HPS400-derived $g_\alpha 3^-$ cells (Pupillo and Devreotes, unpublished data); JH142, JH8-derived $g_\alpha 4^-$ (Hadwiger and Firtel, 1992), JH177, $g_\alpha 4^-$ cells expressing wild-type levels of $G_\alpha 4$, and control kAX3 (provided by J. Hadwiger and R. A. Firtel, University of California, San Diego, CA); LW1, JH10-derived $g_\alpha 7^-$ cells and LW2, a random integrant control; LW3, JH10-derived $g_\alpha 8^-$ cells and LW4, a random integrant control (Wu and Devreotes, unpublished data); JM1 and JM3, MP2 cells overexpressing cAR1 and cAR3, respectively (see below).

AX3 transformants, cAR1/cAR1$^-$, cAR2/cAR1$^-$, cAR3/cAR1$^-$, JM1, and JM3 were grown axenically to a density of 2.5–5 × 10^6 cells/ml in liquid HL5 medium (Watts and Ashworth, 1970) supplemented with 30 μg dihydrostreptomycin/ml and 20 μg Geneticin/ml. Axenic JH177 cultures contained 5 μg Geneticin/ml. AX3, cAR1$^-$, MP2, MP3, LW1, LW2, LW3, and LW4 were cultured under similar conditions in the absence of Geneticin. kAX3 and JH142 were grown in association with *Klebsiella aerogenes* on SM agar plates (Sussman, 1987). Certain strains (JH177, MP3, LW1, LW2, LW3, and LW4) were also grown on plates with bacteria in experiments to measure folate-induced Ca^{2+} entry.

Aggregation-competent amoebas of strains AX3, kAX3, MP3, JH142, LW1, LW2, LW3, and LW4 were obtained by treating the cells for 6–7 h with pulses of exogenous cAMP (Devreotes *et al.*, 1987). JM1 and JM3 cells were starved (22°C) on nonnutrient agar plates as described (Devreotes *et al.*, 1987).

Transformation Procedure

To construct JM1 and JM3 cells, vegetative MP2 amoebas were transformed with pBS18B6 (Klein *et al.*, 1988) or pB18cAR3 (Johnson *et al.*, 1992b) using the procedure of Dynes and Firtel (1989), except that the cells were resuspended in 1 mM Na_2HPO_4/NaH_2PO_4, 250 mM sucrose (pH 6.1), and electroporated using a Bio-Rad (Richmond, CA) Gene Pulser set at 1.2 kV, 200 Ω, and 3 μF (0.2-cm electrode gap cuvette). Amoebas resistant to 20 μg Geneticin/ml were selected as described (Dynes and Firtel, 1989).

Western Blot Analysis

To prepare whole cell extracts for immunoblotting of the cARs, cells (1 × 10^6) washed once in 10 mM KH_2PO_4/Na_2HPO_4 (pH 6.1) were resuspended in sample buffer (Laemmli, 1970) and placed on ice. For immunoblot analysis of $G_\alpha 2$, membranes from cells (5 × 10^6) were prepared as described (Klein *et al.*, 1987), resuspended in sample buffer, and boiled for 5 min. The protein samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gels, transferred electrophoretically to nitrocellulose, visualized using Ponceau S stain to ensure that each lane contained equivalent amounts of protein, and immunoblotted as described (Klein *et al.*, 1987) using cAR1-specific (Klein *et al.*, 1987), cAR3-specific (Johnson *et al.*, 1992a), or $G_\alpha 2$-specific (Gundersen and Devreotes, 1990) antiserum and a [^{125}I]Protein A detection system.

Ca^{2+} Influx Assay

Unless indicated otherwise, amoebas (5 × 10^6) of the desired developmental stage were assayed for chemoattractant-induced $^{45}Ca^{2+}$ uptake as described by Milne and Coukell (1991), except that the assay medium contained 10 μM Ca^{2+}, and folate- and cAMP-induced Ca^{2+} uptake into cells was measured using a 100-μM stimulus. Receptor-

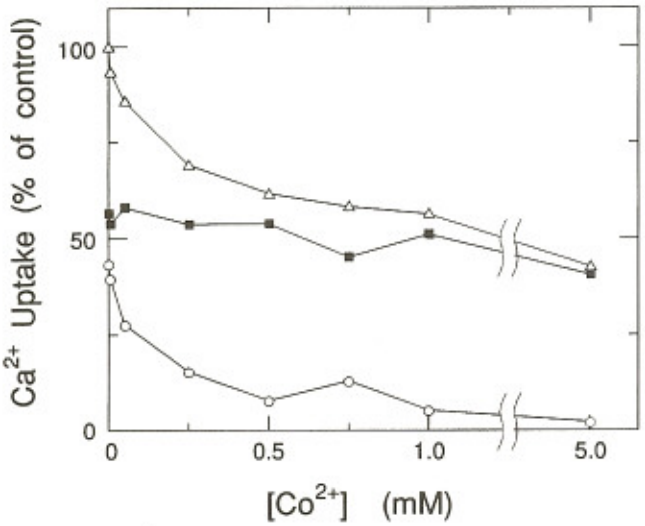

Figure 1. Effect of Co^{2+} concentration on the Ca^{2+} uptake of resting (○) and cAMP-treated (△) cAR3/cAR1⁻ cells. Growth stage amoebas were assayed for 30 s for Ca^{2+} uptake as described in MATERIALS AND METHODS, except that the assay medium was supplemented with increasing concentrations of Co^{2+}. (■) cAMP-stimulated Ca^{2+} uptake. Data from a single experiment are shown, which was repeated once with similar results.

induced Ca^{2+} entry at each timepoint is equal to the amount of Ca^{2+} taken up by stimulated cells minus the amount of Ca^{2+} taken up by resting cells.

Preliminary results indicated that inclusion of $CoCl_2$ in the Ca^{2+} uptake medium increased the sensitivity of the Ca^{2+} uptake assay. Experiments were performed to determine the effect of $CoCl_2$ concentration on the Ca^{2+} uptake of resting and cAMP-stimulated cAR3/cAR1⁻ cells. As shown in Figure 1, Ca^{2+} uptake into nonstimulated cells was inhibited ~5-fold by 0.5 mM $CoCl_2$ and ~14-fold by 5 mM $CoCl_2$. In contrast, the amount of cAMP-stimulated Ca^{2+} uptake remained relatively constant at concentrations of $CoCl_2 < 1$ mM and then declined slightly. Occasionally, 1 mM (but not 0.5 mM) $CoCl_2$ inhibited stimulated Ca^{2+} entry. Therefore, unless indicated otherwise, Ca^{2+}-uptake assays were performed in the presence of 0.5 mM $CoCl_2$. This ion concentration influenced neither the kinetics of cAMP-induced Ca^{2+} uptake nor the sensitivity of this response to stimulus in JM1, JM3, cAR2/cAR1⁻, or cAR3/cAR1⁻ cells.

Additional Assays

[^{3}H]cAMP binding to cells was performed in duplicate using the ammonium sulfate assay of Van Haastert and Kien (1983), except that the final concentration of cAMP in the assay medium was 1 μM.

cAMP-induced accumulation of cGMP (1 μM stimulus) was measured in duplicate using the procedure of Kesbeke *et al.* (1986) and a cGMP [^{125}I] scintillation proximity assay system according to the manufacturer's instructions.

Protein was measured as described by Lowry *et al.* (1951) using bovine serum albumin as standard.

RESULTS

A Ca^{2+}-Influx Pathway can be Activated by Three Distinct Surface cAMP Receptors

When treated with cAMP, AX3 amoebas overexpressing cAR1 in the growth phase initially took up Ca^{2+} at the same rate as nonstimulated cells. After a delay of ~10 s, stimulated Ca^{2+} accumulation rose sharply and continued for 20 s (Figure 2A). This time course is comparable with the cAMP-induced response of wild-type aggregation-competent amoebas (Milne and Coukell, 1991). In contrast, cells transformed with a control plasmid failed to show cAMP-triggered Ca^{2+} entry.

Ca^{2+} uptake was next examined in a cAR1⁻ cell line, which lacks significant surface cAMP binding sites (Sun and Devreotes, 1991), and in cAR1⁻ derived cell lines,

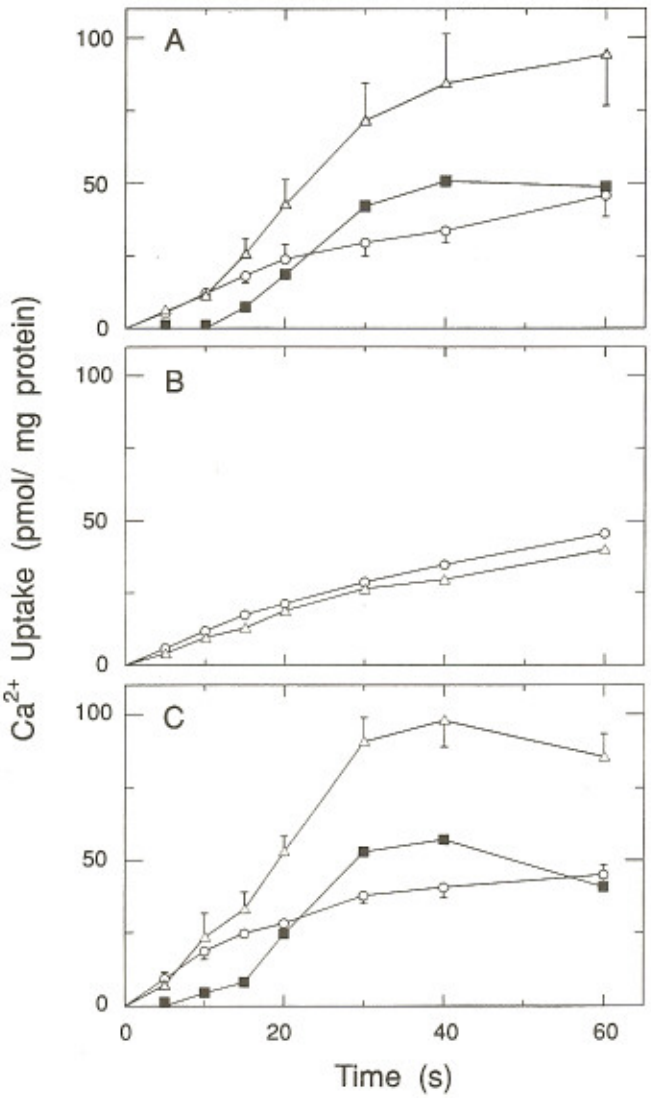

Figure 2. Time course of Ca^{2+} uptake into (A) cAR1-overexpressing AX3 amoebas, (B) cAR1⁻ cells, and (C) cAR1/cAR1⁻ cells. Growth stage amoebas were assayed for Ca^{2+} uptake under standard conditions as described in MATERIALS AND METHODS. Values are shown for Ca^{2+} uptake into resting (○) and cAMP-stimulated cells (△) and for cAMP-induced Ca^{2+} uptake (■). Results shown represent the means of data from (A) three, (B) two, and (C) three separate experiments. In A and C, bars represent SE.

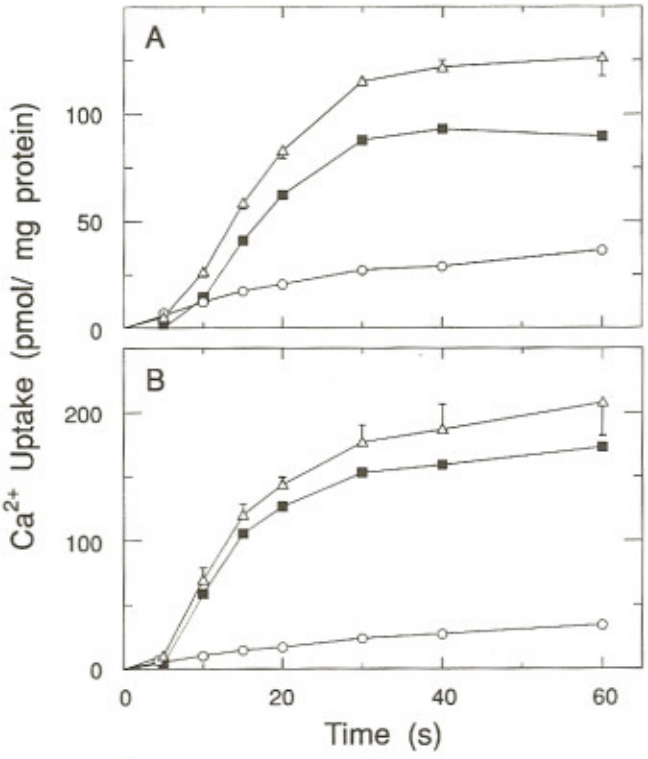

Figure 3. Time course of cAMP-induced Ca^{2+} uptake into (A) cAR2/cAR1$^-$ and (B) cAR3/cAR1$^-$ cells. Growth stage cells were assayed for Ca^{2+} uptake as described in MATERIALS AND METHODS in the absence (○) or presence (△) of cAMP. (■) cAMP-stimulated Ca^{2+} uptake. Values are the means ± SE from (A) five or (B) six separate experiments. (○) Error bars are within the symbol.

which overexpress cAR1, cAR2, or cAR3 (Johnson *et al.*, 1991, 1992b). Growth-phase cAR1$^-$ cells treated with 100 μM cAMP did not accumulate more Ca^{2+} than nonstimulated cells (Figure 2B). Reintroduction of cAR1 into cAR1$^-$ cells (cAR1/cAR1$^-$ cells) restored both surface cAMP binding sites (42 ± 7-fold more sites than cAR1$^-$ cells, mean ± SE, n = 9) and cAMP-induced Ca^{2+} uptake (Figure 2C). The time course and magnitude of stimulated Ca^{2+} entry into cAR1/cAR1$^-$ cells was similar to that of cAR1-overexpressing AX3 cells. cAR1$^-$ cells expressing cAR2 (cAR2/cAR1$^-$ cells) or cAR3 (cAR3/cAR1$^-$ cells) exhibited high levels of surface cAMP binding sites (140 ± 17- and 91 ± 7-fold more sites than cAR1$^-$ cells, respectively, mean ± SE, n = 8). Both strains showed a pronounced cAMP-stimulated Ca^{2+} uptake that had kinetics very similar to the cAR1-induced Ca^{2+} response (compare Figure 3 with Figure 2C) and the folate-induced Ca^{2+} response (Milne and Coukell, 1991).

Properties of cAR2- and cAR3-Mediated Ca^{2+} Influx

The magnitude of receptor-activated Ca^{2+} influx into cAR1$^-$ cells expressing cAR1, cAR2, or cAR3 was dependent on the concentration of cAMP. As illustrated in Figure 4, the Ca^{2+} responses of cAR1/cAR1$^-$ and cAR3/cAR1$^-$ amoebas exhibited similar requirements for cAMP stimulus. Ten to 30 nM cAMP failed to elicit detectable influx, whereas 3–10 μM induced maximal levels of stimulated uptake. In both instances, the concentration of cAMP required for half-maximal Ca^{2+} uptake (EC_{50}) was ~250 nM. In contrast, much higher levels of cAMP (3 μM) were needed to induce stimulated Ca^{2+} uptake in cAR2/cAR1$^-$ cells. Half-maximal and maximal Ca^{2+} uptake occurred at 20 and 300 μM cAMP, respectively. These dose-response curves reflect the relative affinities of the cARs for cAMP. cAR1 and cAR3 possess similar K_d's of ~290 and 490 nM, which are lower than that of cAR2 (K_d > 5 μM) (Johnson *et al.*, 1992b). Stimulated uptake in cAR1/cAR1$^-$ cells was 31 ± 6 pmol Ca^{2+}/mg protein (mean ± SE, n = 5), in cAR2/cAR1$^-$ cells was 90 ± 19 pmol Ca^{2+}/mg protein (mean ± SE, n = 3), and in cAR3/cAR1$^-$ cells was 204 ± 28 pmol Ca^{2+}/mg protein (mean ± SE, n = 4).

To explore whether maximum levels of cAMP-stimulated Ca^{2+} uptake reflected cAR expression levels or whether the cARs differed in their ability to promote Ca^{2+} influx, levels of cAMP-induced Ca^{2+} uptake and of surface cAMP binding sites were measured in various cAR-expressing cell lines. As shown in Table 1, transformants overexpressing cAR1 (cAR1/cAR1$^-$ and JM1 cells) accumulated between 5 and 9 Ca^{2+} ions per binding site. Similar results were obtained with cAR2/cAR1$^-$

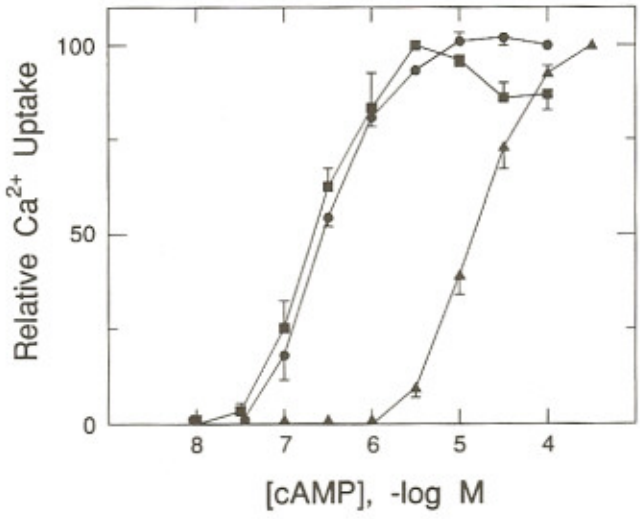

Figure 4. Effect of cAMP concentration on the magnitude of Ca^{2+} uptake into cAR1/cAR1$^-$ (■), cAR2/cAR1$^-$ (▲), and cAR3/cAR1$^-$ (●) cells. Growth stage amoebas were assayed for cAMP-dependent Ca^{2+} uptake as described in MATERIALS AND METHODS, except that uptake was followed for 30 s. Ca^{2+} uptake values for each strain are expressed relative to the maximum uptake value measured in the presence of 3 (■), 100 (●), and 300 (▲) μM cAMP. Each point is the mean ± SE of results obtained in five (■), four (●), and three (▲) independent experiments.

Table 1. Correlation between surface cAMP binding and Ca^{2+} influx in cell lines overexpressing cAR1, cAR2, or cAR3

Strain	Ca^{2+} uptake[a]	cAMP binding[b]	Ca^{2+} uptake/ cAMP binding	n
cAR1/cAR1⁻	37 ± 6	4 ± 1	9.3	9
JM1	54 ± 4	11 ± 2	4.9	6
cAR2/cAR1⁻	88 ± 7	14 ± 2	6.3	9
cAR3/cAR1⁻	154 ± 9	8 ± 2	19.2	9
JM3	58 ± 11	6 ± 1	9.7	5

[a] cAMP-stimulated Ca^{2+} uptake was measured for 30 s as described in MATERIALS AND METHODS. Values are expressed as pmol Ca^{2+} accumulated/mg protein and are the average ± SE of the indicated number of experiments.

[b] Surface cAMP binding was measured as described in MATERIALS AND METHODS. Values are expressed as pmol cAMP binding sites/ mg protein and represent the means ± SE of the indicated number of experiments.

cells and cell lines overexpressing cAR3 (cAR3/cAR1⁻ and JM3 cells).

The similarity between the time course of folate-, cAR1-, cAR2-, and cAR3-mediated Ca^{2+} entry (Figures 2 and 3) (Milne and Coukell, 1991) suggested that cAR2 and cAR3 may activate the same plasma membrane Ca^{2+}-transport system that has been shown to mediate folate- and cAR1-stimulated Ca^{2+} entry (Milne and Coukell, 1991). To test this idea, cAMP-stimulated Ca^{2+} uptake into cAR2/cAR1⁻ and cAR3/cAR1⁻ cells was measured in an assay medium supplemented with compounds known to inhibit by ~50% the folate-induced Ca^{2+} uptake of vegetative amoebas and the cAMP-induced uptake of aggregating cells (Figure 5). The cAMP-mediated Ca^{2+} response of growth stage cAR2/cAR1⁻ and cAR3/cAR1⁻ cells was inhibited by ~50% by 10 μM Ruthenium Red or 2 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP). To determine the ion specificity of the cAR2- and cAR3-triggered Ca^{2+} uptake pathway, competition experiments were performed in the presence of 10 μM Ca^{2+} and 500 μM of various test cations. Stimulated Ca^{2+} entry into cAR2/cAR1⁻ and cAR3/cAR1⁻ cells was not influenced appreciably by Co^{2+}, was inhibited moderately by Cd^{2+}, and was blocked effectively by La^{3+} and Gd^{3+} (Figure 5). Additional experiments revealed that the cAR2- and cAR3-mediated responses were inhibited by 50% (IC_{50}) by ~200–250 μM La^{3+} or Gd^{3+}. This ion selectivity matches that previously reported for the folate- and cAMP-induced Ca^{2+}-uptake systems (Milne and Coukell, 1991). Taken together, these results suggest that the folate receptor, cAR1, cAR2, and cAR3, may couple to a single transporter to regulate Ca^{2+} influx across the plasma membrane.

cAMP-Stimulated Ca^{2+} Entry is Regulated Through a $G_\alpha 2$-Independent Pathway

Recent evidence suggests that folate- and cAMP-mediated signal transduction pathways in *Dictyostelium* involve G proteins (Janssens and Van Haastert, 1987; Van Haastert and Devreotes, 1993). The activation of Ca^{2+} influx by a family of cAMP receptors that resemble known G protein-linked receptors (Klein *et al.*, 1988; Saxe *et al.*, 1991, 1992; Johnson *et al.*, 1992a) suggests that Ca^{2+} entry also may require G protein(s). Previous experiments suggested that the G protein subunit $G_\alpha 1$ regulated neither folate- nor cAMP-induced Ca^{2+} uptake (Milne and Coukell, 1991). To investigate the possible involvement of other G protein subunits in this process, chemoattractant-stimulated Ca^{2+} entry was measured in several G protein null mutants and compared with the Ca^{2+} uptake of appropriate control strains. When $g_\alpha 3^-$, $g_\alpha 4^-$, $g_\alpha 7^-$, or $g_\alpha 8^-$ cells were treated with folate (vegetative amoebas) or cAMP (aggregation-competent amoebas), the kinetics of receptor-induced Ca^{2+} uptake were similar to those of the control cells.

The G protein subunit $G_\alpha 2$ is the likely candidate transducer of cAR1-mediated Ca^{2+} entry because it is preferentially expressed during aggregation (Pupillo *et al.*, 1989) and is critical for several other cAMP-induced responses, including production of cAMP, cGMP, and inositol 1,4,5-trisphosphate (Kesbeke *et al.*, 1988; Snaar-Jagalska *et al.*, 1988). Unexpectedly, it was found that when *Fgd A* mutants HC85 or JH104 (which are defective in the gene encoding for $G_\alpha 2$) (Firtel *et al.*, 1989) were pulsed extensively with cAMP, they exhibited low but reproducible levels of both surface cAMP binding sites and cAMP-stimulated Ca^{2+} entry (Milne and Coukell, 1991).

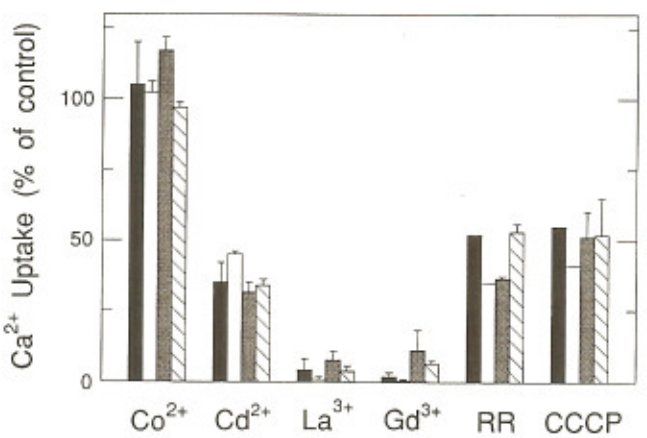

Figure 5. Effect of Ruthenium Red (RR), CCCP, and different cations on receptor-mediated Ca^{2+} uptake. cAMP-induced Ca^{2+} uptake into cAR2/cAR1⁻ cells (stippled bars) and cAR3/cAR1⁻ cells (striped bars) was measured for 30 s as described in MATERIALS AND METHODS, except that the assay medium contained no Co^{2+} and was supplemented 500 μM test cation, 10 μM RR, or 2 μM CCCP. Results are expressed relative to control samples not receiving test compounds and represent the average of four (stippled bars) or three (striped bars) experiments. Bars represent SE. Data depicting the effect of these compounds on folate-induced Ca^{2+} uptake of vegetative cells (closed bars) and cAMP-induced Ca^{2+} uptake of aggregating cells (open bars) were taken from Milne and Coukell (1991).

To investigate further whether $G_\alpha 2$ is a component of the Ca^{2+}-entry system activated by cAR1 or by cAR3 (which is also expressed during aggregation), $g_\alpha 2^-$ cells overexpressing cAR1 or cAR3 were constructed by transformation of $g_\alpha 2^-$ cells with vectors containing cAR1 or cAR3 cDNA under the control of a constitutively active promoter. Stable transformant clones were isolated and screened for receptor expression by immunoblotting with polyclonal cAR1- or cAR3-specific antiserum. Clones exhibiting high levels of cAR1 (JM1 cells) or cAR3 (JM3 cells) were selected for further study.

The expression of cAR1 and cAR3 in JM1 and JM3 cells is illustrated in Figure 6A. Whole cell extracts of growth stage JM1 cells contained a 40-kDa band recognized by cAR1-specific antiserum (lane 3) (Klein *et al.*, 1985). This band, indicative of cAR1 (Klein *et al.*, 1985), was also present in pulsed AX3 cells (lane 1) but not in growth stage $g_\alpha 2^-$ or JM3 cells (lanes 2 and 4). AX3 and JM1 cells also contained a 43-kDa band (Milne and Devreotes, unpublished observation), which likely is the phosphorylated form of cAR1 (Klein *et al.*, 1987). Analysis of the same samples using a cAR3-specific antiserum (lanes 5–8) indicated that JM3 cells (but not JM1, $g_\alpha 2^-$, or 6-h pulsed AX3 cells) contained a 65-kDa band indicative of cAR3 (Johnson *et al.*, 1992a). Additional experiments using a $G_\alpha 2$-specific polyclonal antiserum showed that membrane preparations of JM1 and JM3 cells did not contain detectable levels of $G_\alpha 2$ (lanes 10 and 11), whereas this 40-kDa protein was evident in pulsed AX3 amoebas (lane 9). [^{3}H]cAMP binding studies indicated that vegetative JM1 and JM3 amoebas possessed high levels of surface cAMP binding sites. The former expressed (4.8 ± 0.7, mean ± SE, n = 9) × 10^5 sites/cell, levels that are ~25-fold higher than those of $g_\alpha 2^-$ cells. JM3 cells expressed (3.2 ± 0.5, mean ± SE, n = 5) × 10^5 sites/cell, ~20-fold more than the control cells.

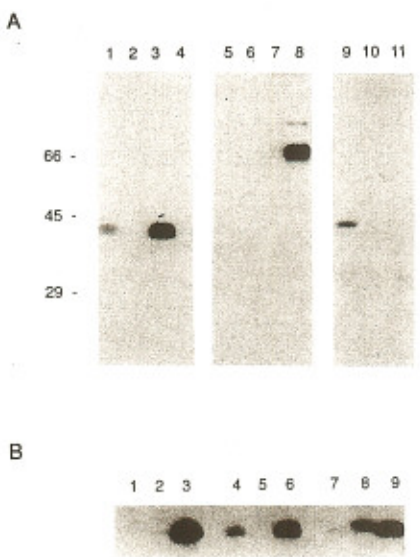

Figure 6. Immunoblot analysis of various *Dictyostelium* strains. (A) Extracts of 6-h pulsed AX3 cells (lanes 1, 5, and 9) and growth stage $g_\alpha 2^-$ (lanes 2 and 6), JM1 (lanes 3, 7, and 10), and JM3 (lanes 4, 8, and 11) cells were prepared, fractionated, and immunoblotted using antibodies to cAR1 (lanes 1–4), cAR3 (lanes 5–8), and $G_\alpha 2$ (lanes 9–11) as described in MATERIALS AND METHODS. Numbers at the margin of the figure indicate the migration position of molecular weight standards expressed in kDa. (B) Developmental expression of cAR1 in AX3 (lanes 1, 4, and 7), $g_\alpha 2^-$ (lanes 2, 5, and 8), and JM1 (lanes 3, 6, and 9) cells. Amoebas treated with exogenous cAMP pulses were harvested at 0 h (lanes 1–3), 5.5 h (lanes 4–6), or 11 h (lanes 7–9), and cell lysates were prepared and analyzed by immunoblotting with cAR1-specific antiserum as described in A.

The regulation of cAR1 in suspensions of developing AX3, JM1, and $g_\alpha 2^-$ cells is shown in Figure 6B. In AX3 cells, cAR1 expression reached a maximum at 5.5 h of starvation and declined at 11 h (lanes 1, 4, and 7), consistent with the findings of Klein *et al.* (1987). Growth stage JM1 cells expressed very high levels of cAR1, which declined slightly as the cells entered development and then remained constant (lanes 3, 6, and 9). In contrast, little or no detectable cAR1 was evident in $g_\alpha 2^-$ cells until between 5.5 and 11 h of starvation (lanes 2, 5, and 8).

Evidence suggests that cAMP-mediated regulation of certain effector enzymes is defective in $g_\alpha 2^-$ cells starved for ~5–6 h (Kesbeke *et al.*, 1988; Kumagai *et al.*, 1991). However, because early developing $g_\alpha 2^-$ amoebas express only low levels of cAR1 (Kesbeke *et al.*, 1988) (Figure 6B), we sought to substantiate the role of $G_\alpha 2$ in these signal transduction events. Accordingly, JM1 and JM3 cells were used to demonstrate a stringent requirement for $G_\alpha 2$. For example, overexpression of cAR1 or cAR3 in $g_\alpha 2^-$ cells did not restore normal cellular development on phosphate-buffered agar. Both strains remained aggregation deficient even after prolonged incubation. JM1 cells did not exhibit cAR1-mediated activation of adenylate cyclase (Pupillo *et al.*, 1992). In addition, cGMP production was compared in growth stage JM1 cells and in aggregation stage AX3 cells. Although both strains showed high levels of cAMP binding sites, only AX3 cells displayed cAMP-stimulated cGMP production (30 pmol/mg protein 10 s after cAR activation, mean of values from 2 experiments).

Both JM1 and JM3 cells did show a cAR1-mediated Ca^{2+} entry during growth phase (Figure 7,A and B) or after 5 h of starvation (Milne and Devreotes, unpublished observation), which possessed kinetics comparable with the cAMP-induced response of aggregation-competent wild-type cells (Milne and Coukell, 1991). The magnitude of stimulated Ca^{2+} uptake in both strains was similar to the cAMP-induced Ca^{2+} uptake of other cAR1- or cAR3-overexpressing cells (Table 1). In contrast, growth stage $g_\alpha 2^-$ cells did not exhibit cAMP-stimulated Ca^{2+} entry (Figure 7C).

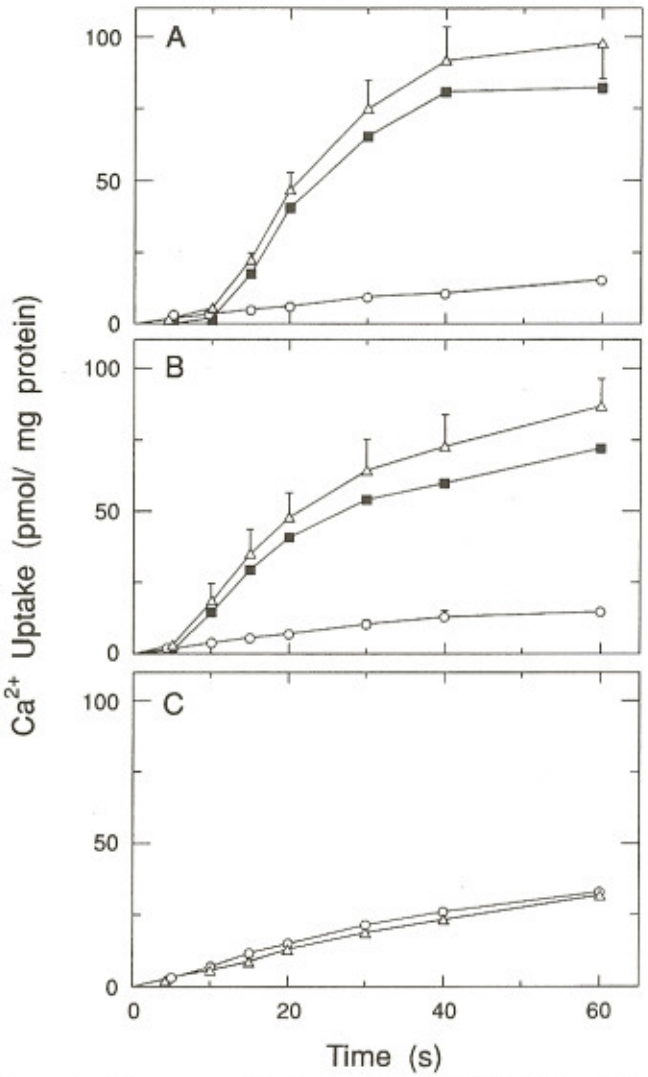

Figure 7. Time course of Ca^{2+} entry into (A) JM1 (B) JM3 and (C) $g_\alpha 2^-$ cells. Growth stage cells were assayed for Ca^{2+} uptake in the absence (○) or presence (△) of cAMP as described in MATERIALS AND METHODS. (■) cAMP-stimulated Ca^{2+} uptake. Results represent the means of data from (A) four, (B) five, and (C) two separate experiments. In A and B, bars represent SE. (○) Error bars are within the symbol.

DISCUSSION

We have used a sensitive Ca^{2+}-influx assay to investigate chemoreceptor-mediated signal transduction in *Dictyostelium*. Recent evidence indicates that folate (in growth stage cells) and cAMP (in aggregation stage cells) activate a similar Ca^{2+}-entry pathway (Milne and Coukell, 1991). Our characterization of cAR1$^-$ cells overexpressing cAR1, cAR2, or cAR3 suggests that cARs normally present during multicellular development can promote Ca^{2+} influx. Moreover, our findings suggest that cAR2 and cAR3 activate the same Ca^{2+}-transport system as that regulated by folate receptors during growth phase and cAR1 during aggregation. For example, the kinetics of Ca^{2+} influx mediated by each receptor appeared to be remarkably similar (Figures 2 and 3) (Milne and Coukell, 1991). Each Ca^{2+}-influx system was inhibited ~50% by 10 μM Ruthenium Red and 2 μM CCCP (Figure 5), compounds that may act by depolarizing the plasma membrane and reducing the driving force for Ca^{2+} entry (Milne and Coukell, 1991). Because membrane depolarization is known to activate voltage-gated channels of mammalian cells (Tsien *et al.*, 1987), these findings, together with the observations that these compounds do not alter the time course of folate- or cAR1-mediated Ca^{2+} entry (Milne and Coukell, 1991), suggest that each of the cARs and the folate receptor stimulate Ca^{2+} entry through a voltage-independent mechanism. This idea is supported by the findings that cAR1- and folate-induced Ca^{2+} uptake is not inhibited by high concentrations of the classical Ca^{2+}-channel blockers diltiazem, nifedipine, nicardipine, methoxyverapamil, or verapamil (Milne and Coukell, 1991). Finally, ion competition experiments revealed that each receptor activated a Ca^{2+}-entry system that was highly selective for the transport of Ca^{2+} over other di- and trivalent cations (Figure 5). Importantly, the cation blockade profile of the Ca^{2+}-influx pathway is different from that of *Dictyostelium* Ca^{2+}-adenosine triphosphatase pumps, which are inhibited effectively by low micromolar concentrations of La^{3+} and Cd^{2+} (Milne and Coukell, 1989).

The cAMP concentration dependence of cAR1-, cAR2-, and cAR3-stimulated Ca^{2+} influx suggests that these responses are directly proportional to the fraction of occupied receptors. The dose-response profiles of cAMP-stimulated Ca^{2+} entry into growth stage cAR1/cAR1$^-$ and cAR3/cAR1$^-$ cells were similar with EC_{50}'s of ~200–250 nM (Figure 4). Under physiological cAMP binding conditions, the majority of surface binding sites on cAR1- and cAR3-overexpressing strains have comparable affinities with K_d's of ~230 and 490 nM, respectively (Johnson *et al.*, 1992b). In contrast, the cAR2-overexpressing cells required much higher concentrations of cAMP for stimulated Ca^{2+} entry (EC_{50} = 20 μM) (Figure 4), consistent with the observation that these cells exhibit little detectable surface binding even at 5 μM [^{3}H]cAMP (Johnson *et al.*, 1992b). Although the threshold stimulus required to induce Ca^{2+} entry varied among the cARs, they promoted ion influx with similar effectiveness (5–20 Ca^{2+} ions/receptor) in the presence of saturating levels of cAMP (Table 1).

Analogous to higher eukaryotic systems, receptor-mediated changes in the amount and distribution of cellular Ca^{2+} in *Dictyostelium* likely play a critical role in chemotaxis, cell differentiation, and morphogenesis (see references in Milne and Coukell, 1988; Coukell and Cameron, 1988; Van Duijn and Van Haastert, 1992). In this study, cAR1-, cAR2-, and cAR3-activated Ca^{2+}

uptake was characterized in growth stage cells. However, it is probable that these receptors regulate Ca^{2+} fluxes when expressed in their normal physiological context. In aggregating cells, a cAMP stimulus leads to an influx of extracellular Ca^{2+} (Wick *et al.*, 1978; Bumann *et al.*, 1984; Milne and Coukell, 1991). cAR1, the major cAMP receptor present during aggregation, likely regulates Ca^{2+} influx during this developmental stage. However, cAR1 levels decline after 6–8 h of starvation (Klein *et al.*, 1987), whereas cAMP-induced Ca^{2+} uptake remains constant until ≥14 h of development (Milne and Coukell, 1991). Ca^{2+} influx also occurs in intact slugs (Kuhtreiber and Jaffe, 1990), which accumulate higher levels of Ca^{2+} in prestalk cells than in prespore cells (Maeda and Maeda, 1973). These Ca^{2+} fluxes may be regulated by cAR3 and cAR2, which are maximally expressed in mound stage and slug stage cells, respectively (Johnson *et al.*, 1992a; Saxe *et al.*, 1992). It remains to be determined whether the Ca^{2+}-influx system is activated by cAR4, which is also present during multicellular development (Louis, Ginsburg, and Kimmel, personal communication).

The regulation of Ca^{2+} influx in *Dictyostelium* by a family of cARs that resemble known G protein receptors (Klein *et al.*, 1988; Saxe *et al.*, 1991, 1992; Johnson *et al.*, 1992a) suggests that this response may involve G proteins. The best characterized cAR, cAR1, appears to act through the G protein α subunit $G_\alpha 2$ to regulate cAMP-induced synthesis of cAMP, cGMP (Kesbeke *et al.*, 1988), and inositol 1,4,5-trisphosphate (Snaar-Jagalska *et al.*, 1988). Surprisingly, cAMP was found to induce a small Ca^{2+} influx in repeatedly stimulated $g_\alpha 2^-$ cells (Milne and Coukell, 1991). However, the role of $G_\alpha 2$ in cAMP-mediated Ca^{2+} entry could not be evaluated in this initial study because the stimulated Ca^{2+} uptake was small and $g_\alpha 2^-$ cells express low levels of cAR1. Analysis of growth stage JM1 cells, which express considerably higher levels of cAR1 than stimulated $g_\alpha 2^-$ cells (Figure 6B), revealed that the time course and magnitude of stimulated Ca^{2+} influx in this strain was comparable with the Ca^{2+} response of wild-type aggregation-competent amoebas (compare Figure 7 with Figure 1 in Milne and Coukell, 1991). This finding indicates that $G_\alpha 2$ is not required for cAR1-induced Ca^{2+} entry. In contrast, overexpression of cAR1 in JM1 cells failed to restore several $G_\alpha 2$-dependent responses, including cAMP-induced stimulation of adenylate cyclase (Pupillo *et al.*, 1992) and guanylate cyclase and cell aggregation.

Together, these results show that cAR1 activates certain effectors through $G_\alpha 2$ but it triggers Ca^{2+} influx through a $G_\alpha 2$-independent mechanism. It seems likely that different affinity states of cAR1 mediate $G_\alpha 2$-dependent and -independent signal transduction. [^{3}H]cAMP binding studies with cAR1-overexpressing cells show that ~25% of the surface cAMP binding sites possess an affinity of 30 nM, whereas the remainder possess an affinity of 230 nM (Johnson *et al.*, 1992b). The high-affinity state of cAR1 appears to regulate both adenylate and guanylate cyclase activities, which are responsive to low nanomolar cAMP (Mato *et al.*, 1977a; Theibert *et al.*, 1986). In contrast, the low affinity form of cAR1 may be necessary for the Ca^{2+} response, which is elicited by much higher stimulus concentrations in both wild-type aggregating cells (Milne and Coukell, 1991) and in growth stage cAR1/cAR1$^-$ cells (Figure 4). Our results with JM3 cells indicate that cAR3 can also activate normal Ca^{2+} influx through a $G_\alpha 2$-independent pathway. The cAMP dose dependency of cAR3-induced Ca^{2+} entry (Figure 4) suggests that the low affinity form of cAR3 (Johnson *et al.*, 1992b) also activates this response.

The functional domains of the cARs required for cAMP binding and for the binding and activation of G proteins remain to be defined. These receptors share considerable homology in the transmembrane-spanning regions, which may constitute the cAMP binding site, based on analogy to the β-adrenergic receptor and rhodopsin (Johnson *et al.*, 1992a). Evidence from mammalian systems indicates that the ability of seven transmembrane domain receptors to interact with G proteins depends on the second and third cytoplasmic loops and a postulated fourth loop that arises by the insertion into the membrane of palmitate linked to cysteine residue(s) of the C-terminal domain (reviewed in Hargrave, 1991). Short stretches (~9–20) of amino acids in the C-terminal and N-terminal regions of the third intracellular loop appear to be important in determining the specificity of receptor/G-protein interactions (Kobilka *et al.*, 1988; Lechleiter *et al.*, 1990; Okamoto *et al.*, 1991). Little is known about how G proteins couple to the *Dictyostelium* cARs, although the second and third cytoplasmic loops share extensive amino acid identity (Johnson *et al.*, 1992a). This similarity suggests that a single G protein or a family of related G proteins may be involved in signal transduction through these receptors. However, it cannot be excluded that the few divergent amino acids in these regions may switch the specificity of G protein/receptor coupling.

It is unclear which G protein(s), if any, regulates the Ca^{2+}-influx system. Characterization of null mutants of various G protein α subunits indicates that folate- and cAMP-triggered Ca^{2+} entry does not require $G_\alpha 1$ (Milne and Coukell, 1991), $G_\alpha 2$, $G_\alpha 3$, $G_\alpha 4$, $G_\alpha 7$, or $G_\alpha 8$. Moreover, although the role of $G_\alpha 5$ and $G_\alpha 6$ in receptor-activated Ca^{2+} entry is not yet established, the expression profiles of these α subunits during development (Hadwiger *et al.*, 1991; Wu and Devreotes, 1991) imply that neither may fulfill this function. It is possible that functionally redundant G proteins expressed at distinct times during development couple to the four chemoreceptors. However, we do not favor this idea because the eight α subunits do not appear to group into distinct subclasses (Wu *et al.*, 1992). In addition, although $g_\alpha 2^-$ cells express $G_\alpha 1$ and $G_\alpha 3$ (Pupillo and Devreotes, per-

sonal communication), and likely $G_\alpha 6$ and $G_\alpha 8$ (Wu and Devreotes, 1991), these G-protein subunits do not compensate for the absence of $G_\alpha 2$ and permit activation of $G_\alpha 2$-dependent effector enzymes.

Certain mammalian $G_{\beta\gamma}$ complexes have been shown to regulate downstream effectors, including ion channels (Jelsema and Axelrod, 1987; Logothetis *et al.*, 1987; Tang and Gilman, 1991; Katz *et al.*, 1992). *Dictyostelium* amoebas possess a single G_β subunit that is highly homologous to those of mammalian cells (Pupillo *et al.*, 1988). We are currently determining whether receptor-mediated Ca^{2+} entry requires G proteins using recently constructed g_β null cells (Lilly, Wu, Welker, and Devreotes, personal communication). If the cAR1-mediated Ca^{2+} response persists in these cells, then it would strongly reinforce our hypothesis that these seven transmembrane domain receptors can transduce certain signals independently of G proteins.

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