Dissection of the cAMP induced cytosolic calcium response in *Dictyostelium discoideum*: the role of cAMP receptor subtypes and G protein subunits

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Abstract The cAMP signaling cascade leading to changes in $[Ca^{2+}]_i$ in *Dictyostelium discoideum* was analyzed using cell lines overexpressing single cAMP receptor subtypes (cAR1–cAR3) or lacking the $G_{\alpha 2}$ or G_β subunit of the G protein. Imaging of fura2-dextran-loaded amoebae revealed cAMP-induced $[Ca^{2+}]_i$ changes characteristic for each receptor subtype activated. Cells expressing distinct subtypes sort to defined zones during multicellular development suggesting involvement of the specific $[Ca^{2+}]_i$ transients in patterning processes. Whereas generation of the $[Ca^{2+}]_i$ increase was $G_{\alpha 2}$-independent, only few cells devoid of G_β displayed a $[Ca^{2+}]_i$ change after stimulation indicating its participation in the regulation of the calcium homeostasis.

Key words: Development; Signal transduction; $G_{\alpha 2}$; G_β; Fura2-dextran; $[Ca^{2+}]_i$ imaging

1. Introduction

During the asexual life cycle of the cellular slime mould *Dictyostelium discoideum* cAMP triggers chemotactic aggregation of the amoebae and is involved in their further differentiation into two cell types, i.e. spores and stalk cells. cAMP binds to cell surface receptors of which four subtypes (cAR1–cAR4) are known. These are expressed differentially throughout development, both temporally as well as spatially in the multicellular stages; in addition, their affinities for cAMP are different.

cAR1 is the receptor expressed predominantly during early differentiation and is responsible for chemotaxis [1]; it has two binding sites of rather high affinities for cAMP (K_D 25 and 230 nM, respectively) [2]. At later stages of development besides low levels of cAR1 the subtypes cAR3 and cAR2 are expressed [3,4]. cAR3 also has two binding sites for cAMP with affinities that are in the range of that of cAR1 (K_D 14 and 490 nM); the affinity of cAR2 for cAMP is much lower with a K_D of >5 μM [2]. The other low affinity receptor cAR4 is expressed only very late during development [5,6]. cAR1 is expressed in all cells during aggregation [7]. cAR2 and cAR3 appear to be colocalized during late aggregation [8]; in the following pseudoplasmodium stage cAR2 is expressed in the anterior prestalk cell zone, whereas cAR3 is restricted to the posterior prespore cell region with almost no overlap [8,9]. cAR4 expression occurs at the tip of the pseudoplasmodium in the prestalk zone and in the posterior region in the 'anterior like cells' [5].

The signaling cascade triggered by cAMP binding to cAR1 is well characterized and leads to changes in the calcium homeostasis of the amoebae which is necessary for their chemotactic migration [10]. The activated receptor couples to a heterotrimeric G protein whose α subunit is $G_{\alpha 2}$; among the 8 α subunits which are also expressed differentially throughout development the $G_{\alpha 2}$ as well as the single G_β subunit are essential for multiple responses during cAMP signal transduction leading to chemotactic aggregation (for review see [7]). G protein dependent activation of phospholipase C [11–13] leading to formation of IP_3 as well as of phospholipase A_2 [14] results in release of stored Ca^{2+} and Ca^{2+} influx [15–17]. Following a cAMP stimulus cGMP is synthesized [18,19] and participates in the control of Ca^{2+} entry across the plasma membrane [16,20]. Ca^{2+} influx has been measured in cell suspensions by $^{45}Ca^{2+}$ uptake [21] and with Ca^{2+}-sensitive electrodes [17]. Recently, cAMP-induced $^{45}Ca^{2+}$ uptake was shown to be independent of the type of receptor activated: cell lines overexpressing either cAR1, cAR2 or cAR3 all responded with maximum influx at cAMP concentrations of 3–300 μM, corresponding to the affinity of the receptors; the amount of Ca^{2+} uptake per cAMP binding site was shown to differ between the subtypes [22]. Moreover, part of the influx was reported to be independent of the $G_{\alpha 2}$ or the G_β subunit of the G protein [22–24].

After cAR1 stimulation the amount of Ca^{2+} influx should lead to a corresponding $[Ca^{2+}]_i$ change in the range of 10–20 μM [17], yet $[Ca^{2+}]_i$ elevations measured in single cells with the fluorescent indicators fura2, fura2-BSA or fura2-dextran were rather small, i.e. only up to ca. 150 nM above basal levels [25–27]. Suspension measurements of aequorin-transfected cells also revealed but small $[Ca^{2+}]_i$ transients [28,29]. Obviously amoebae possess an efficient buffering capacity that enables them to carefully restrict the cAMP-induced $[Ca^{2+}]_i$ changes resulting from release of stored Ca^{2+} or from Ca^{2+} influx temporally and/or spatially.

The influence of stimulation of other cAMP receptor subtypes or of G protein subunits on the $[Ca^{2+}]_i$ homeostasis is not known. The aim of this study was to characterize the cAMP-triggered signaling cascade leading to $[Ca^{2+}]_i$ changes with respect to the role of different receptor subtypes as well as the participation of a functional G protein. We tested whether stimulation of cell lines expressing either distinct cAMP receptors or lacking the $G_{\alpha 2}$ or G_β subunit of the G protein evoked $[Ca^{2+}]_i$ transients. We characterized the cAMP induced $[Ca^{2+}]_i$ responses of these strains and compared them with respect to (i) the percentage of cells exhibiting a $[Ca^{2+}]_i$ elevation and (ii) to its size. This allowed to obtain informa-

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Abbreviation: $[Ca^{2+}]_i$, cytosolic free calcium concentration

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tion regarding the underlying signal transduction mechanism(s). Activation of each receptor subtype induced a characteristic $[Ca^{2+}]_i$ change. The generation of the $[Ca^{2+}]_i$ increase was independent of $G_{\alpha 2}$; in contrast, most of the cells lacking G_β did not respond, indicating a requirement of G_β for proper regulation of calcium homeostasis.

2. Materials and methods

2.1. Materials

Fura2 and fura2-dextran were from MobiTec (Göttingen, Germany); cAMP was from Boehringer (Mannheim, Germany). [^{3}H]cAMP was from Amersham (Braunschweig, Germany); specific radioactivity was 26 Ci/mmol.

2.2. Culture of cells

Wild type Ax2 was grown as described [27]. Cell lines expressing distinct cAMP receptor subtypes were grown in plastic dishes containing medium supplemented with 20 μg/ml G418 and 100 μg/ml ampicillin at 23°C. Strains used were: control strain devoid of cAR1 and cAR3 (cAR1$^-$/cAR3$^-$; [30]) termed RI9, RI9 transformed with wild-type cAR1 [30], cAR1$^-$ cell lines (Δ208; [1]) expressing cAR2 or cAR3 (cAR2/Δ208, cAR3/Δ208; [2]), $G^-_{\alpha 2}$ cells overexpressing cAR1 (JM1; [22]) and G^-_β cell lines expressing either cAR1 or cAR3 (LW14 and LW17, respectively; [23]). Development was induced by washing cells free of medium by centrifugation (3×) and resuspension in ice-cold Sørensen phosphate buffer (SP-buffer: 17 mM (KH_2/Na_2H)-PO_4, pH 6.0). Cell density was adjusted to 2×10^7 cells/ml; the suspension was shaken at 150 rpm and 23°C. Time in hours after the induction of development is designated t_x.

2.3. $[Ca^{2+}]_i$ measurements

Cells were loaded with the Ca^{2+}-indicator fura2-dextran at $t_{0.5}$ by electroporation or with fura2 by scrape-loading exactly as described [27,31]. $[Ca^{2+}]_i$ measurements were done at $t_{1.5-5}$ in H5-buffer (5 mM KCl, 5 mM HEPES, pH 7.0)+1 mM $CaCl_2$; $[Ca^{2+}]_i$ responses did not change within this time period. After transfer to the stage of an inverted microscope IM or AxiovertT100 (Zeiss, Jena, Germany) the amoebae were viewed with a 100× Ultrafluar (NA 1.25) or 100× Fluar (NA 1.3) objective (Zeiss). Stimulation was carried out by adding a drop of cAMP (10 μl) to the medium (90 μl). $[Ca^{2+}]_i$ imaging was performed by dual excitation at 340 and 380 nm with a filter wheel and a 50 W mercury arc lamp and grey filters or a 100 W AttoArc system (Zeiss) set at an output of 1%; fluorescence images were captured with an ICCD camera (RKH-80, Heimann, Wiesbaden or HL-A, Proxitronic, Bensheim, Germany) as described [25,27]. The amount of indicator incorporated was similar in the different strains used in this study; from comparison of the fluorescence intensity of the cells it was in the range of that reported for wild-type Ax2 [27]. The fluorescence intensity at 340 nm excitation and before stimulation, at a fixed camera setting, was 122 ± 36, 125 ± 29 and 138 ± 33 for cAR1-, cAR3- and cAR2-expressing cells (grey values; mean ± S.E.M. of 60, 187 and 95 cells, respectively). The grey values of JM1, LW14 and LW17 were 86 ± 2, 116 ± 27 and 117 ± 26 (mean ± S.E.M. of 179, 121 and 142 cells, respectively). Ratioing of the fura2 signals renders the determination of $[Ca^{2+}]_i$ independent of the indicator concentration. Therefore, variations in the $[Ca^{2+}]_i$ response were not due to differential uptake of dye.

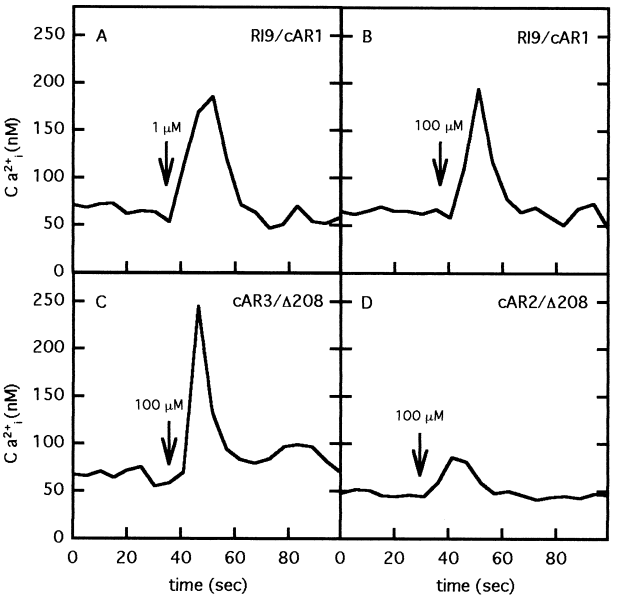

Fig. 1. Dependence of the $[Ca^{2+}]_i$-change on the subtype of cAMP-receptor stimulated. Single cAR1$^+$ amoebae (RI9/cAR1) were challenged with 1 or 100 μM cAMP (A,B). cAR3$^+$ (cAR3/Δ208; C) and cAR2$^+$ amoebae (cAR2/Δ208; D) were stimulated with 100 μM cAMP. Time point of addition is marked by an arrow.

2.4. cAMP binding assay

cAMP binding was analyzed in the presence of ammonium sulfate as described [2,32]. Binding was tested by adding 10^7 cells at $t_{0.25-0.5}$ to 100 μl SP buffer that was supplemented with 10 nM [^{3}H]cAMP, 1 μM cAMP and 10 mM dithiothreitol. Cells were incubated for 1 min at 0°C. Subsequently, 850 μl ammonium sulfate (3 M) and 50 μl bovine serum albumin (10 mg/ml) were added. After further incubation for 5 min and centrifugation for 3 min the cell pellet was dissolved in formic acid and the radioactivity was counted. Assays were run in duplicate. Non-specific binding was determined by incubation with 1 mM cAMP before addition of ammonium sulfate.

3. Results

3.1. Activation of distinct cAMP receptor subtypes elicits transient $[Ca^{2+}]_i$ changes

We used genetically modified strains to test whether the $[Ca^{2+}]_i$ increase observed in wild-type cells [27] requires concomitant activation of different cAMP receptor subtypes or whether activation of single subtypes suffices to evoke a response. Strain RI9/cAR1 expresses cAR1 but is deficient of cAR3 [30]; in this study it will be termed cAR1$^+$. Strains cAR2/Δ208 and cAR3/Δ208, termed cAR2$^+$ and cAR3$^+$, respectively, lack cAR1 [2]. cAR2$^+$ overexpresses the subtype

Table 1
Influence of the type of cAMP-receptor stimulated on the percentage of responding cells, the height of the $[Ca^{2+}]_i$ increase and on basal $[Ca^{2+}]_i$

Cell line:	RI9/cAR1			cAR3/Δ208			cAR2/Δ208		
cAMP receptor subtype:	cAR1			cAR3			cAR2		
cAMP (μM):	0.1	1	100	0.1	1	100	1	100	1000
Reacting cells	42%	33%	46%	84%	76%	87%	15%	12%	10%
(total number of cells tested)	(36)	(123)	(345)	(73)	(212)	(398)	(270)	(512)	(182)
$[Ca^{2+}]_i$ change (nM; mean ± S.E.M., from responding cells only)	86 ± 11	127 ± 10	138 ± 7	103 ± 5	169 ± 7	162 ± 4	60 ± 5	65 ± 5	74 ± 11
$[Ca^{2+}]_i$ basal (nM; mean ± S.E.M.)		63 ± 1			61 ± 1			58 ± 1	

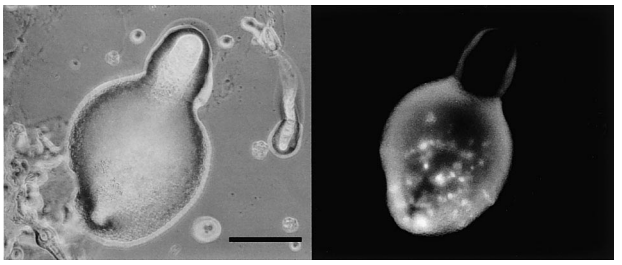

Fig. 2. Localization of cAR3$^+$ in multicellular structures. cAR3$^+$ cells labeled with FITC-dextran by scrape-loading were mixed with untreated wild-type Ax2 in a ratio of 1:2 and allowed to develop. Fluorescent cells are in the posterior prespore zone but not in the anterior tip. Phase contrast (left) and fluorescence image (right). Bar: 100 μm.

cAR2 but is devoid of cAR3 whereas cAR3$^+$ overexpresses the cAR3 receptor but has no cAR2 [2,3]. As a control we tested the cAR1$^-$/cAR3$^-$ strain RI9 that completely lacks cAMP responsiveness [33]; in this strain cAMP never induced a $[Ca^{2+}]_i$ change (not shown) indicating requirement of receptor activation to elevate $[Ca^{2+}]_i$. Fig. 1 shows $[Ca^{2+}]_i$ of single cells expressing distinct receptor subtypes that were loaded with fura2-dextran by electroporation. Immediately after cAMP addition a transient $[Ca^{2+}]_i$ rise occurred that lasted 20–30 s. The height of the response in cAR1$^+$ cells was similar when stimulated with 1 μM or 100 μM cAMP (Fig. 1A,B). Under these conditions binding approaches saturation (K_D of cAR1: 25 and 230 nM [2]) and $^{45}Ca^{2+}$ uptake was found to be roughly equal [22]. Stimulation with 100 μM cAMP also evoked a $[Ca^{2+}]_i$ transient in cAR3$^+$ amoebae (Fig. 1C) but exceeding that of cAR1$^+$ cells; the same result was obtained when lower cAMP concentrations were applied. The $[Ca^{2+}]_i$ elevation of cAR2$^+$ was small as compared to the other cell lines (Fig. 1D). In general, the time course of the $[Ca^{2+}]_i$ response was similar between the strains.

The behaviour of the cells was analyzed with respect to (i) the percentage of amoebae displaying a $[Ca^{2+}]_i$ increase, (ii) the mean height of the $[Ca^{2+}]_i$ change and (iii) basal $[Ca^{2+}]_i$. Table 1 gives the quantitation of these parameters. cAR3$^+$ cells displayed the most pronounced responses. The percentage of cells reacting with a $[Ca^{2+}]_i$ change was high, in the range of 76–87%, irrespective of the dose of cAMP applied (0.1–100 μM). The mean height of the $[Ca^{2+}]_i$ rise in cAR3$^+$ cells (K_D of cAR3: 14 and 490 nM [2]) was similar when 1 or 100 μM cAMP were added. At lower cAMP doses the $[Ca^{2+}]_i$ change was smaller. The fraction of responding cAR1$^+$ cells was lower, in the range of 33–46%, again independent of the concentrations of cAMP tested (0.1–100 μM). The height of the $[Ca^{2+}]_i$ elevation was significantly lower in cAR1$^+$ than in cAR3$^+$ (138 ± 7 nM, mean ± S.E.M., as compared to 162 ± 4 nM at 100 μM cAMP; Mann Whitney's rank sum test: $P < 0.001$). This difference in the height of the $[Ca^{2+}]_i$ change is in agreement with the higher amount of $^{45}Ca^{2+}$ uptake observed in cAR3$^+$ than in cAR1$^+$ after cAMP stimulation [22]. As observed in cAR3$^+$ amoebae the mean height of the $[Ca^{2+}]_i$ increase in cAR1$^+$ cells was comparable when 1 or 100 μM cAMP was added but smaller after application of 0.1 μM cAMP. In addition, the two strains were not different with respect to basal $[Ca^{2+}]_i$ which was 61–63 nM. Compared to both of these strains, $[Ca^{2+}]_i$ responses of cAR2$^+$ cells (low affinity cAMP receptor; $K_D > 5$ μM [2]) were drastically altered following stimulation with 1 μM, 100 μM or 1 mM cAMP. The percentage of responding cells (10–15%) and the mean height of the $[Ca^{2+}]_i$ rise (60 ± 5–74 ± 11 nM; mean ± S.E.M.) were significantly lowered in cAR2$^+$ cells (Mann Whitney's rank sum test: $P < 0.001$). This difference in the response between cAR2$^+$ and cAR3$^+$ was also observed when cells scrape-loaded with fura2 were analyzed (not shown). Basal $[Ca^{2+}]_i$ was determined to be 58 ± 1 nM. In contrast to $[Ca^{2+}]_i$ imaging, no $^{45}Ca^{2+}$-influx was found in cAR2$^+$ cells when 1 μM cAMP was added [22]. The difference between the data is most likely due to the experimental conditions: influx was measured at an external $[Ca^{2+}]$ of 10 μM whereas $[Ca^{2+}]_i$ was determined in the present study at 1 mM $[Ca^{2+}]_e$. Presumably under these conditions Ca^{2+} influx is rather high in all strains and also occurs in cAR2$^+$ resulting in $[Ca^{2+}]_i$ changes. At 10 μM $[Ca^{2+}]_e$ practically no $[Ca^{2+}]_i$ increase was observed even in wild-type cells, although significant influx occurs [17] which is most probably due to rapid sequestration. In fact a $[Ca^{2+}]_e$ of = 100 μM is required to induce full $[Ca^{2+}]_i$ changes [26,29]. Obviously then the high degree of Ca^{2+} influx overcomes the sequestration systems of the cells.

Expression of the cAMP receptor subtypes in the cell lines is under the control of the same promoter and constitutive during early development [3]. Yet, the observed effects might still be due to variations in the level of receptor expression between the strains and/or between cells within the same strain. Indeed we found that the number of cAMP binding sites per cell was different between cAR1$^+$, cAR3$^+$ and cAR2$^+$ ($4.2 \pm 0.8 \times 10^5$, $5.2 \pm 0.6 \times 10^5$ and $1.5 \pm 0.3 \times 10^5$, respectively; mean ± S.E.M. from 4 experiments each) and, within this range, variable from day to day. However, in experiments where receptor expression was comparable between strains the same characteristic percentages of responding cells were observed (e.g. 40% of responding cAR1$^+$ cells having 5×10^5 binding sites/cell vs. 90% of response in cAR3$^+$ cells with 4×10^5 binding sites/cells). In addition, even when the number of cAMP binding sites/cell was different by a factor

Table 2
Effect of the presence of a functional G protein on the percentage of responding cells, the $[Ca^{2+}]_i$ change evoked by cAMP stimulation and basal $[Ca^{2+}]_i$

Cell line:	JM1	LW14	LW17
G-protein subunit lacking:	$G_{\alpha 2}$	G_β	G_β
cAMP receptor subtype:	cAR1	cAR1	cAR3
Reacting cells (total number of cells tested)	59% (462)	7% (232)	20% (632)
$[Ca^{2+}]_i$ increase (nM; mean ± S.E.M.)	113 ± 4	86 ± 10	114 ± 6
$[Ca^{2+}]_i$ basal (nM; mean ± S.E.M.)	64 ± 1	59 ± 3	61 ± 1

Cells were stimulated with 100 μM cAMP; in two experiments cAMP binding sites were determined to be $4 \pm 1.7 \times 10^5$ sites/cell in JM1, $7 \pm 1.3 \times 10^5$ sites/cell in LW14 and $2.5 \pm 0.1 \times 10^5$ sites/cell in LW17. The mean of the $[Ca^{2+}]_i$ change is from the responding cells only.

of two, e.g. between a pair of experiments with cAR1$^+$ amoebae, the fraction of reacting cells and the height of the $[Ca^{2+}]_i$ increase was similar. Moreover, challenge of cAR2$^+$ with low doses of cAMP where the receptors are not saturated, i.e. at 1 μM, was sufficient to induce a $[Ca^{2+}]_i$ elevation in 15% of the cells. The height already amounted to 80% of that elicited after addition of 1 mM cAMP. Surprisingly, in two experimental batches 90% and 100% of cAR2$^+$ cells responded (as compared to the mean of 15% and 12% after 1 μM or 100 μM cAMP, respectively). The magnitude of the $[Ca^{2+}]_i$ change was similar to that found in experiments where only 12% and 15% of the cells had reacted.

As cAR3 and cAR2 are expressed differentially in multicellular stages of development [8] we tested whether the overexpressing cell lines sort to distinct sites when allowed to develop together with wild-type Ax2. Indeed, cAR3$^+$ labeled with FITC-dextran was observed in posterior regions of multicellular structures only (Fig. 2); localization in anterior zones or in terminally differentiated stalks did not occur (at least 20 pseudoplasmodia, culminants and fruiting bodies each were analyzed in 4 independent experiments). Fluorescent cAR2$^+$ cells were not found in multicellular stages of development (not shown); they did not aggregate both in the absence or presence of wild-type Ax2 but instead remained as single cells on the substratum. Obviously, the aggregation defect of this strain, probably due to the lack of cAR1, cannot be overcome by the presence of wild-type cells. Although wild-type Ax2 amoebae synthesize and secrete cAMP during aggregation the resulting concentrations are too low to activate the low affinity receptors of cAR2$^+$ amoebae.

3.2. *Requirement of the $G_{\alpha 2}$- or G_β-subunit of the G protein for the $[Ca^{2+}]_i$ rise*

We have shown previously that challenge of vegetative HC85 cells that lack $G_{\alpha 2}$ with high doses of cAMP led to a $[Ca^{2+}]_i$ increase with characteristics similar to vegetative wild-type Ax2 [25]; yet these cells show only low cAMP binding [34]. Now we used strain JM1 that is devoid of $G_{\alpha 2}$ but overexpresses cAR1 [22]. The $[Ca^{2+}]_i$ change of a single JM1 cell after addition of 100 μM cAMP is shown in Fig. 3A. The cells also responded to 1 μM cAMP with no difference in the characteristics (not shown) which is in agreement with the cAMP-binding properties of cAR1 [2]. When compared to cAR1$^+$ cells the reaction of JM1 was slightly smaller (113 ± 4 vs. 138 ± 7 nM; mean ± S.E.M.) yet more cells responded (Fig. 3A and Table 2). These data reveal that the $[Ca^{2+}]_i$ rise in JM1 is independent of $G_{\alpha 2}$.

The requirement for the G_β-subunit to induce $[Ca^{2+}]_i$ changes was tested using the strains LW14 and LW17 that are devoid of a functional β subunit and overexpress cAR1 or cAR3, respectively [23]. Challenge with saturating cAMP doses of 100 μM evoked $[Ca^{2+}]_i$ elevations in both cell lines that were smaller than in the corresponding G_β^+ strains. Their height was comparable to the transients induced in cAR1$^+$ and cAR3$^+$ cells after stimulation with 0.1 μM cAMP (Fig. 3B,C and Table 2). In contrast to the G_β^+ strains, in both LW14 and LW17 the percentage of responding amoebae was rather low, 7 and 20%, respectively (Table 2). The same low percentage of reacting LW14 cells was observed when cells scrape-loaded with fura2 were investigated (2 positive out of 87 tested). We conclude that G_β, either directly or indirectly, is involved in the generation of the $[Ca^{2+}]_i$ transient.

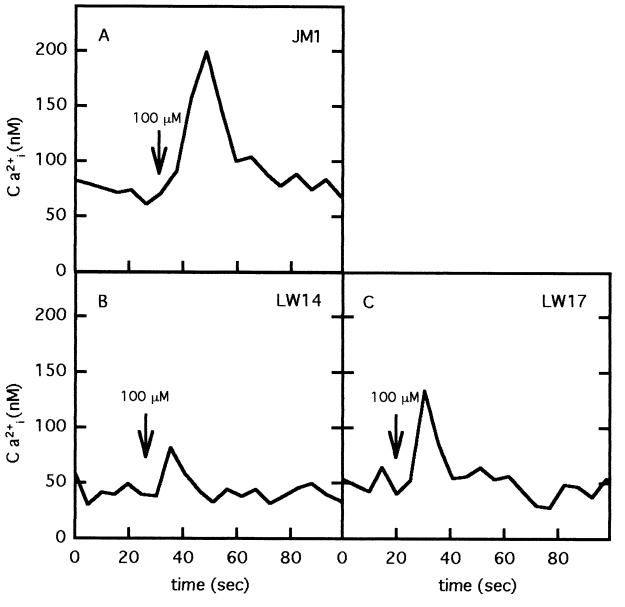

Fig. 3. Effect of the presence of a functional G-protein on the cAMP-induced $[Ca^{2+}]_i$ increase. Single $G_{\alpha 2}^-$ cell overexpressing cAR1 (JM1; A) or cells lacking G_β and overexpressing either cAR1 (LW14; B) or cAR3 (LW17; C) were challenged with 100 μM cAMP. Time point of addition is marked by an arrow.

4. Discussion

$[Ca^{2+}]_i$ transients induced by cAMP in *D. discoideum* could arise from simultaneous activation of different receptor subtypes. Alternatively, stimulation of each of the subtypes could be sufficient to evoke a $[Ca^{2+}]_i$ elevation. Our results show that this is the case. Yet in developing wild type concomitant cAMP activation of several receptor types is likely. During aggregation different subtypes are present on the cell surface. cAR1 expression is maximal and cAR3 mRNA is also detected already at t_6 [3]. Although cAR2 mRNA was undetectable until at least t_{10} [4] very low amounts of the protein could be present.

Each of the strains expressing distinct cAMP receptor subtypes displayed characteristic responses: $[Ca^{2+}]_i$ changes were strongest in cAR3$^+$ cells, lowest in cAR2$^+$ and of intermediate height in cAR1$^+$ amoebae. In addition, the fraction of reacting cells was clearly different between the strains. One could suspect that these characteristics were simply due to variations in the level of receptor expression between strains or between cells within one strain. In fact, the mean numbers of receptors determined by cAMP binding assays were different by a factor of 3.5 between these strains. Yet, there are several lines of evidence that argue against this notion: in experiments where the number of binding sites was comparable between different strains the same characteristic percentages of responding cells were observed. The height of the $[Ca^{2+}]_i$ increase and the percentage of responding cells was not altered when the level of receptor expression, e.g. in cAR1$^+$, varied by a factor of two between pairs of experiments. In two experimental batches, the percentage of responding cAR2$^+$ was high, 90% and 100%; the height of the $[Ca^{2+}]_i$ transient was the same as that observed when

only 10–15% of amoebae responded. This argues for the presence of sufficient receptors in all cells. Moreover, in all strains occupancy of only a part of the receptors available resulted in the same fraction of responding cells as after stimulation with saturating cAMP doses. Challenge of $cAR2^+$ with 1 μM cAMP should result in binding of 20% of receptors since there is one low affinity cAR2 binding site ($K_D > 5$ μM) [2]. Under these conditions 15% of the cells responded with a $[Ca^{2+}]_i$ increase that amounted to 80% of that after addition of 1 mM cAMP. This indicates that there is a threshold number of receptors that have to be activated in order to induce a $[Ca^{2+}]_i$ increase and that stimulation of more receptors does not significantly augment the $[Ca^{2+}]_i$ rise. Taken together, the data support the view that the $[Ca^{2+}]_i$ responses do not result from different numbers of binding sites/cell in the strains or from the level of receptor expression within the cells of one strain. Instead, activation of distinct receptor types leads to defined characteristics of the $[Ca^{2+}]_i$ change with respect to the percentage of responding cells and the height of the response.

Our results imply that the presence of a particular receptor type leads to a specific physiologic state of the cell. This possibly reflects differentiation processes occurring under normal, wild-type conditions. Specificity is gained by differential regulation of the $[Ca^{2+}]_i$ homeostasis with respect to agonist-induced changes. Yu and Saxe [8] found that cAR2 and cAR3 are cell type specific receptors present preferentially in prestalk or prespore cells, respectively. In line with these data our mixing experiments revealed sorting of $cAR3^+$ cells to the prespore region of pseudoplasmodia but not to the prestalk zone. The developmental pathways of prestalk and prespore cells involve different regulation of the $[Ca^{2+}]_i$ homeostasis which we showed to be controlled by the type of cAMP receptor present on the cell surface. In prespore cells activation of cAR3 could lead to large $[Ca^{2+}]_i$ changes whereas predominant stimulation of cAR2 would induce only small $[Ca^{2+}]_i$ elevations in prestalk amoebae. This supports a model proposed earlier that suggested both lower basal $[Ca^{2+}]_i$ and agonist induced $[Ca^{2+}]_i$ changes in prestalk compared to prespore cells due to a higher degree of Ca^{2+} sequestration ([35], for review see [36]). Indeed, higher amounts of sequestered and also of total Ca^{2+} in prestalk than in prespore cells were reported [37,38]. Yet, the conclusions that can be drawn from the $^{45}Ca^{2+}$-uptake results of Milne and Devreotes [22] and our $[Ca^{2+}]_i$ imaging data are opposite to earlier findings on $[Ca^{2+}]_i$ regulation in prestalk and prespore cells. Using amoebae expressing aequorin cDNA constructs in the cytosol, smaller $[Ca^{2+}]_i$ transients were found in suspensions of dissociated prespore cells compared to prestalk cells when stimulated with 50 nM cAMP at 10°C [28]. In addition, higher aequorin luminescence was found in the anterior region of the pseudoplasmodium in situ leading to the conclusion of higher $[Ca^{2+}]_i$ levels in the prestalk zone [39]. These contrasting views require further investigations as in the multicellular stages of development additional clues besides cAMP come from the activation of other signaling cascades by effectors such as DIF-1 (for review see [36,40]). The effect of combinations of cAMP and DIF-1 on $[Ca^{2+}]_i$ regulation is currently being studied.

Results obtained with JM1 revealed that the signaling cascade activated by cAR1 leading to $[Ca^{2+}]_i$ transients does not require the $G_{\alpha 2}$ subunit of the G protein. Responses of JM1 were similar to $cAR1^+$; JM1 shows a somewhat higher percentage of reacting cells but a slightly lower height of the $[Ca^{2+}]_i$ change.

The necessity of the G_β subunit of the G protein to generate a $[Ca^{2+}]_i$ transient was investigated using LW14 and LW17 cells overexpressing cAR1 and cAR3, respectively [23]. Stimulation with 100 μM cAMP led to a $[Ca^{2+}]_i$ increase in LW14 as well as in LW17 which was, however, smaller than in the corresponding G_β^+ strains. Rather, the height of the $[Ca^{2+}]_i$ transients in LW14 and LW17 was similar to those evoked in the G_β^+ cells after stimulation with 0.1 μM cAMP. This is in accordance with $^{45}Ca^{2+}$-influx data or electrode recordings which show that both in LW14 and LW17 cAMP-activated Ca^{2+} influx was reduced and only roughly 20% of the influx of $cAR1/G_\beta^+$ and $cAR3/G_\beta^+$ or of wild-type amoebae; in the G_β^+ strains influx after addition of 0.1 μM cAMP also amounted to 20% compared to 100 μM cAMP [22–24]. However, in contrast to $cAR1/G_\beta^+$ and $cAR3/G_\beta^+$, most of the LW14 cells (93%) displayed no $[Ca^{2+}]_i$ change and only 20% of LW17 amoebae responded. These results indicate that a functional G_β-subunit is involved in the cAMP-activated generation of the $[Ca^{2+}]_i$ rise.

This could be accomplished in two ways. (i) G_β could take part in the clearance of Ca^{2+} from the cytosol. In hepatocytes inhibition of a plasma membrane Ca^{2+}ATPase by $G_{\beta\gamma}$ was described [41]. If the same regulation occurred in *Dictyostelium*, lack of G_β would result in a constitutively active Ca^{2+} pump rapidly removing Ca^{2+} from the cytosol; Ca^{2+} influx would only lead to a local $[Ca^{2+}]_i$ rise beneath the plasma membrane. In *D. discoideum* Ca^{2+} pumps located in the plasma membrane or in intracellular compartments have both high affinities for Ca^{2+} in the submicromolar range as well as high transport capacity, resulting in effective clearance of Ca^{2+} from the cytosol already at basal levels of 50 nM [15,42–44]. In case of a fully active pump, Ca^{2+} entering the cell could be directly pumped out again leading to diminished uptake observed both in LW14 and LW17. This assumption seems realistic based on comparison of the activity of the plasma membrane Ca^{2+} ATPase [42] and the $^{45}Ca^{2+}$ flux data [22,23]. (ii) Alternatively, $G_{\beta\gamma}$ might influence, either directly or indirectly, coupling of the receptor to plasma membrane Ca^{2+} channels. In rat pituitary GH_3 cells loss of receptor-mediated regulation of the Ca^{2+} channel was achieved by injection of the antisense oligonucleotide β-com that hybridized with the mRNA of specific β-subunits [45].

A puzzling aspect of cAMP signaling is the fact that clonal cell populations exhibit heterogeneity. Only a certain percentage of the amoebae responded with a $[Ca^{2+}]_i$ rise while the rest remained 'silent' (this gets averaged out, of course, when suspension measurements are performed), even when growth stage overexpressors of cAMP receptors were analyzed. Interestingly, in *D. discoideum* Ca^{2+} and pH oscillations occur with a period length of 7–8 min [46]. Within these oscillations cells exhibit excitable and refractory phases [47]. A possibility to explain the above finding of heterogeneity is that cAR1, cAR2 and cAR3 overexpressors differ in their respective lengths of the excitable phase. $cAR2^+$ cells might be activated only during a short time interval which would be longer in $cAR1^+$ and $cAR3^+$ amoebae. As cAR2 is present on prestalk cells only [8] the reduced phase of excitability might serve to sharpen the oscillations, which might help prestalk cells to function as pacemakers [48,49].

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References

[1] Sun, T.J. and Devreotes, P.N. (1991) Genes Dev. 5, 572–582.
[2] Johnson, R.L., Van Haastert, P.J.M., Kimmel, A.R., Saxe, C.L., Jastorff, B. and Devreotes, P.N. (1992) J. Biol. Chem. 267, 4600–4607.
[3] Johnson, R.L., Saxe, C.L., Gollop, R., Kimmel, A.R. and Devreotes, P.N. (1993) Genes Dev. 7, 273–282.
[4] Saxe, C.L., Ginsburg, G.T., Louis, J.M., Johnson, R.L., Devreotes, P.N. and Kimmel, A.R. (1993) Genes Dev. 7, 262–272.
[5] Louis, J.M., Ginsburg, G.T. and Kimmel, A.R. (1994) Genes Dev. 8, 2086–2096.
[6] Ginsburg, G.T. and Kimmel, A.R. (1997) Genes Dev. 11, 2112–2123.
[7] Parent, C.A. and Devreotes, P.N. (1996) Annu. Rev. Biochem. 65, 411–440.
[8] Yu, Y. and Saxe, C.L. (1996) Dev. Biol. 173, 353–356.
[9] Saxe, C.L., Yu, Y., Jones, C., Bauman, A. and Haynes, C. (1996) Dev. Biol. 174, 202–213.
[10] Unterweger, N. and Schlatterer, C. (1995) Cell Calcium 17, 97–110.
[11] Kumagai, A., Pupillo, M., Gundersen, R., Miake-Lye, R., Devreotes, P.N. and Firtel, R.A. (1989) Cell 57, 265–275.
[12] Europe-Finner, G.N. and Newell, P.C. (1987) J. Cell Sci. 87, 221–229.
[13] Van Haastert, P.J.M., Devries, M.J. and Penning, L.C. et al. (1989) Biochem. J. 258, 577–586.
[14] Schaloske, R. and Malchow, D. (1997) Biochem. J. 327, 233–238.
[15] Europe-Finner, G.N. and Newell, P.C. (1986) Biochim. Biophys. Acta 887, 335–340.
[16] Flaadt, H., Jaworski, E., Schlatterer, C. and Malchow, D. (1993) J. Cell Sci. 105, 255–261.
[17] Bumann, J., Wurster, B. and Malchow, D. (1984) J. Cell Biol. 98, 173–178.
[18] Mato, J., Krens, F., Van Haastert, P.J.M. and Konijn, T.M. (1977) Proc. Natl. Acad. Sci. USA 74, 2348–2351.
[19] Wurster, B., Schubiger, K., Wick, U. and Gerisch, G. (1977) FEBS Lett. 76, 141–144.
[20] Menz, S., Bumann, J., Jaworski, E. and Malchow, D. (1991) J. Cell Sci. 99, 187–191.
[21] Wick, U., Malchow, D. and Gerisch, G. (1978) Cell Biol. Int. Rep. 2, 71–79.
[22] Milne, J.L. and Devreotes, P.N. (1993) Mol. Biol. Cell 4, 283–292.
[23] Milne, J.L.S., Wu, L.J., Caterina, M.J. and Devreotes, P.N. (1995) J. Biol. Chem. 270, 5926–5931.
[24] Schaloske, R., Sordano, C., Bozzaro, S. and Malchow, D. (1995) J. Cell Sci. 108, 1597–1603.
[25] Schlatterer, C., Gollnick, F., Schmidt, E., Meyer, R. and Knoll, G. (1994) J. Cell Sci. 107, 2107–2115.
[26] Yumura, S., Furuya, K. and Takeuchi, I. (1996) J. Cell Sci. 109, 2673–2678.
[27] Sonnemann, J., Knoll, G. and Schlatterer, C. (1997) Cell Calcium 22, 65–74.
[28] Saran, S., Nahao, H. and Tasaka, M. et al. (1994) FEBS Lett. 337, 43–47.
[29] Nebl, T. and Fisher, P.R. (1997) J. Cell Sci. 110, 2845–2853.
[30] Kim, J.-Y., Soede, R.D.M. and Schaap, P. et al. (1997) J. Biol. Chem. 272, 27313–27318.
[31] Schlatterer, C., Knoll, G. and Malchow, D. (1992) Eur. J. Cell Biol. 58, 172–181.
[32] Van Haastert, P. and Kien, E. (1983) J. Biol. Chem. 258, 9636–9642.
[33] Kim, J.-Y., Caterina, M.J., Milne, J.L.S., Lin, K.C., Borleis, J.A. and Devreotes, P.N. (1997) J. Biol. Chem. 272, 2060–2068.
[34] Milne, J.L. and Coukell, M.B. (1991) J. Cell Biol. 112, 103–110.
[35] Gross, J.D., Peacey, M.J. and Pogge von Strandmann, R. (1988) Differentiation 38, 91–98.
[36] Gross, J.D. (1994) Microbiol. Rev. 58, 330–351.
[37] Maeda, Y. and Maeda, M. (1973) Exp. Cell Res. 82, 125–130.
[38] Tirlapur, U.K., Gross, J. and Nanjundiah, V. (1991) Differentiation 48, 137–146.
[39] Cubitt, A.B., Firtel, R.A., Fischer, G., Jaffe, L.F. and Miller, A.L. (1995) Development 121, 2291–2301.
[40] Newell, P.C., Malchow, D. and Gross, J.D. (1995) Experientia 51, 1155–1165.
[41] Lotersztain, S., Pavoine, C. and Deterre, P. et al. (1992) J. Biol. Chem. 267, 2375–2379.
[42] Böhme, R., Bumann, J., Aeckerle, S. and Malchow, D. (1987) Biochim. Biophys. Acta 904, 125–130.
[43] Milne, J.L. and Coukell, M.B. (1989) Exp. Cell Res. 185, 21–32.
[44] Rooney, E.K., Gross, J.D. and Satre, M. (1994) Cell Calcium 16, 509–522.
[45] Kleuss, C., Scherübl, H., Hescheler, J., Schultz, G. and Wittig, B. (1992) Nature 358, 424–426.
[46] Bumann, J., Malchow, D. and Wurster, B. (1986) Differentiation 31, 85–91.
[47] Malchow, D., Nanjundiah, V. and Gerisch, G. (1978) J. Cell Sci. 30, 319–330.
[48] Weijer, C., McDonald, S. and Durston, A. (1984) Differentiation 28, 9–12.
[49] Nanjundiah, V. and Saran, S. (1992) J. Biosci. 17, 353–394.