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Intracellular free calcium level and its response to cAMP stimulation in developing *Dictyostelium* cells transformed with jellyfish apoaequorin cDNA

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Abstract

A new method is described for measuring intracellular free calcium concentrations, $[(Ca^{2+})_i]$, in the cells of *Dictyostelium discoideum* transformed with apoaequorin cDNA of the jellyfish, *Aequorea victoria*. Aequorin, a calcium-specific indicator, was regenerated in vivo from apoaequorin produced in the cells by incubation with coelenterazine. The results showed that $[(Ca^{2+})_i]$ in developing cells markedly increases at the aggregation stage and again at the culmination stage after a temporary drop at the migration stage. Except for the vegetative stage, the cells at all stages of development exhibit a sharp transient increase in $[(Ca^{2+})_i]$ upon stimulation with a cAMP (50 nM) pulse, high responses being observed at the migration and culmination stages. Separated prestalk cells of migrating slugs contain more than twice as much $[(Ca^{2+})_i]$ and show three times as large a response to cAMP stimulation as prespore cells.

Key words: *Dictyostelium discoideum*; Aequorin; Cyclic AMP; Calcium; Cell differentiation

1. Introduction

The cellular slime mold, *Dictyostelium discoideum*, is an ideal system for the study of cell differentiation and pattern formation in multicellular development. Free living amoebae of this organism grow and multiply until the food is depleted. Starvation triggers the onset of multicellular development and cells aggregate at common collecting points by chemotaxis towards cAMP. Interaction of cAMP with cell surface receptors activates the intracellular signal transduction pathways leading to chemotactic movement and cAMP amplification and relay [1]. At the center of aggregation, a long and thin migrating slug or a pseudoplasmodium soon develops which has an anterior 20% made of prestalk cells and a posterior 80% made of prespore cells. The slug culminates to form a mature fruiting body, consisting of a mass of spores (from the prespore cells) and a supporting stalk made of vacuolated cells (from the prestalk cells).

Calcium ion has been proposed to play a key role as a second messenger in numerous cellular processes in this organism, such as cell differentiation and movement. In response to cAMP, an increase in cytosolic calcium is believed to be involved in chemotactic response [2,3], polymerization of cytoskeletal actin [4], stimulation of plasma membrane ATPase [5], and developmental gene expression [6,7]. Calcium oscillations [8,9] may also be involved in cell-to-cell signalling via wave propagation within a slug. Calcium is also known to be involved in differentiation of the prestalk and prespore cells within the slug. Using atomic absorption measurements, Maeda and Maeda [10] showed that the total cellular calcium concentration is higher in the anterior prestalk cells of the slug than in the posterior prespore cells. Tirlapur et al. [11] have shown higher calcium sequestration in the prestalk cells using chlortetracycline, a probe for sequestered calcium. Direct measurements of intracellular free calcium, $[(Ca^{2+})_i]$, and its transient changes in response to the chemoattractant, cAMP, was made by Abe and Maeda [12] using the calcium indicators, Fura-2/AM and Quin-2/AM. Their results suggest involvement of intracellular calcium as a signal transmitter in chemotactic responses. One of the major difficulties of using the calcium indicators, however, is that *Dictyostelium* cells are quite impermeable to them. Therefore, in order to load the indicators into the cells, electroporation must be used

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which might cause damage to the cells and possibly make them release sequestered calcium into the cytosol. In fact, it has been reported that approximately 30% of the cells die after electroporation [2].

In the present study, we describe a new method which overcomes the difficulties associated with the previous method for the measurement of cytosolic free calcium [13]. We allowed *Dictyostelium* cells to express jellyfish apoaequorin cDNA and the protein produced is regenerated in vivo into aequorin by incubating the cells with coelenterazine. Aequorin, a photoprotein found in the jellyfish, *Aequorea victoria*, consists of a complex of apoaequorin (apoprotein), coelenterazine (substrate) and molecular oxygen. When it is treated with calcium ions, the calcium-triggered oxidation of coelenterazine yields coelenteramide, and the excited state of coelenteramide bound to apoaequorin emits a blue light (λ_{max} = 470 nm) [15].

In the present work, intact *Dictyostelium* cells expressing the apoaequorin cDNA were used to monitor changes in intracellular free calcium content, $[(Ca^{2+})_i]$, and its response to exogenous cAMP during development and differentiation.

2. Materials and methods

2.1. Construction of apoaequorin cDNA expression plasmid

The plasmid piQ5 containing the apoaequorin cDNA has been described [13]. The expression plasmid pDQ12 was constructed by inserting the 0.6 kb *Sal*I–*Eco*RI apoaequorin cDNA fragment of the piQ5 into a modified *Eco*RI site of the pDNeoII vector containing the actin 6 promoter and the actin 8 terminator of *Dictyostelium*. Fig. 1 illustrates the pDQ12 construct.

D. discoideum cells of strain Ax-2 were transformed with pDQ12 by electroporation and the transformants were selected on G418 medium. The transformants were analysed for the expression of apoaequorin by immunoblotting using anti-aequorin rabbit antibody and peroxidase-tagged anti-rabbit IgG. A clone of the transformants with appropriate apoaequorin expression was selected for further experiments.

2.2. Measurement of intracellular free calcium

Calcium-independent in vivo luminescence was measured essentially according to the method of Nakajima-Shimada et al. [16], with slight modifications. Exponentially growing cells (4–8 × 10^6 cells/ml) were harvested and washed in 0.1 M MES-Tris buffer, pH 6.5 (Sol A). The cells were suspended in 30 μl of Sol A containing 50 μM coelenterazine and incubated for 30 min at 22°C under shaken conditions. The cells were washed in Sol A, transferred to a cuvette with a magnetic stirrer, and the initial light intensity was measured at 10°C. (The temperature for measurement was lowered to 10°C because the response to cAMP stimulation was too rapid at room temperature.) To relate the amplitude of the recorded aequorin signals to the amount of aequorin actually present in the cells, experiments were terminated with 0.5% Triton X-100 for recording the light emitted when all the aequorin in the preparation was suddenly discharged [17].

For developmental studies, exponentially growing cells were allowed to develop on filters to the desired stage, and intracellular free calcium was monitored as described above. Migrating slugs were washed, collected on nylon meshes and a Percoll gradient centrifugation was performed for the separation of prestalk and prespore cells [18]. Intracellular free calcium was monitored in the same way.

2.3. Calibration of aequorin signal

The aequorin luminescence intensity was calibrated in a solution (100 mM KCl, 10 mM MOPS, potassium salt, pH 7.2) by using Ca-EGTA buffers [19] according to Nakajima-Shimada et al. [16]. A calibration curve was drawn relating free calcium concentration to the ratio of peak light intensity to the maximum light intensity measured at a saturating calcium concentration [17]. Intracellular free calcium was estimated from the ratio of aequorin light intensity of the *Dictyostelium* cells (L) to the maximum light intensity after the Triton treatment (L_{max}). Ax-2 cells loaded with coelenterazine was substracted as a blank value from all the results.

3. Results

3.1. Estimation of cytosolic calcium in vegetative cells

The $[(Ca^{2+})_i]$ level at the vegetative stage was found to be 100 nM. It was the lowest level to be found throughout development (Table 1).

Very high luminescence intensity was generated when 10 μM A23187 + 10 mM $CaCl_2$ was added to intact *Dictyostelium* cells containing apoaequorin cDNA which had been preincubated with coelenterazine and washed in a calcium-deficient buffer (Fig. 2). To rule out the possibility that the observed luminescence transients were due to leakage of aequorin into the medium, supernatants separated both before and after the addition of the stimulants were assayed for calcium-dependent luminescence. The results showed no detectable luminescence from either sample (data not shown). No response was observed when pulses of 50 nM cAMP were given to the vegetative cells.

3.2. Changes in cytosolic calcium during development

Cells from different developmental stages showed characteristic differences in their levels of $[(Ca^{2+})_i]$, as well as their response to cAMP (Table 1). There is a sharp increase (> 2.5-fold) in $[(Ca^{2+})_i]$ when the vegeta-

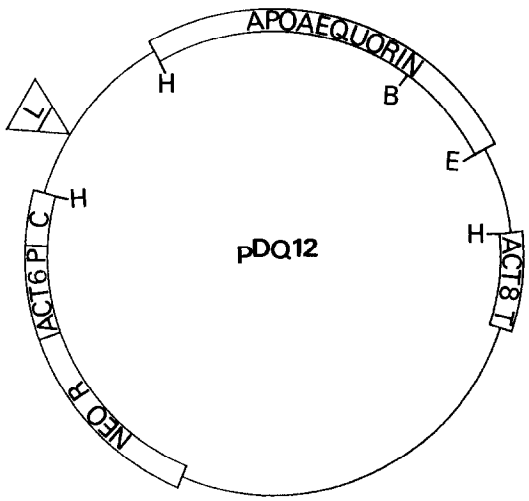

Fig. 1. Structure of pDQ12 which was constructed by inserting the 0.6 kb apoaequorin cDNA of piQ5. pDQ12 contains the promoter (Act 6 P) and the first 8 codons (C) of Actin 6 gene of *Dictyostelium*. A linker (L) was inserted to build an in-frame construct, and the transcriptional terminator was derived from the Actin 8 gene. H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI.

tive cells were allowed to form loose aggregates. This level drops down to 130 nM at the migrating slug stage, and the decrease is followed by an increase of approximately 2-fold at the early culmination stage.

As the amount of apoaequorin expressed in transformed cells, as estimated by the Western blots using anti-aequorin, was found to be equal at all the stages of development (data not shown), the difference in the emitted light must reflect the difference in the level of $[(Ca^{2+})_i]$.

In response to pulses of 50 nM cAMP, a small but significant transient increase in $[(Ca^{2+})_i]$ levels was observed with aggregative cells (Fig. 3). Such a response becomes remarkable at the migrating slug stage, resulting in about a 2-fold increase in $[(Ca^{2+})_i]$. The high responsiveness still remains at the early culmination stage, although to a lesser extent. cAMP pulses at intervals of 5 min were given beforehand to 'prime' the cells for the detection of the response (see section 4). At no stage of development did a single pulse of 50 nM cAMP to the cells give any measurable response.

3.3. $[(Ca^{2+})_i]$ levels in prestalk and prespore cells and their response to cAMP

To examine whether or not prestalk and prespore differentiation in migrating slugs is reflected in any difference in $[(Ca^{2+})_i]$ and its responsiveness to exogenous cAMP, the cell types were separated by Percoll density gradient centrifugation as described in section 2. The degree of contamination was 2–5% in each cell sample type as examined by antispore immunoglobulin. Measurements of $[(Ca^{2+})_i]$ in each cell type showed that the intracellular free calcium is approximately 2.5 times higher in the prestalk cells than in the prespore cells (Table 2). The responses of these two cell types to cAMP stimulation were markedly different; 'primed' prestalk cells exhibited a considerable increase (1.5-fold) in $[(Ca^{2+})_i]$ with a 50 nM cAMP pulse, while the prespore cells showed a smaller response upon stimulation, comparable to that of the aggregative cells (Fig. 4A and B; Table 2). In both cases, the response is very rapid and the maximum level is reached within ca. 5 s (at 10°C).

Table 1

Intracellular free calcium concentration, $[(Ca^{2+})_i]$, in developing cells of *Dictyostelium discoideum* and its increase upon stimulation with cAMP

Developmental stage	$[(Ca^{2+})_i]$ ± S.D. (nM)	
	Resting level	Increase upon cAMP (50 nM) stimulation
Vegetative	100 ± 32.34	No increase
Aggregative	264 ± 37.91	42 ± 2.64
Slug	127 ± 25.17	132 ± 2.83
Early culmination	243 ± 30.59	194 ± 17.31

All measurements were made at 10°C. $n = 3–5$.

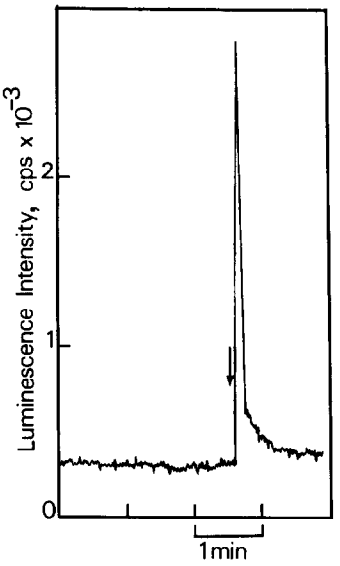

Fig. 2. Effect of $CaCl_2$ + A23187 on the luminescence activity of intact *D. discoideum* cells carrying the pDQ12 plasmid. The arrow indicates the point of addition of $CaCl_2$ + A23187.

The increase, however, is followed by a gradual decrease in the case of prestalk cells.

4. Discussion

The present method for the measurement of $[(Ca^{2+})_i]$ is characteristic in that it uses apoaequorin produced in intact *Dictyostelium* cells transformed with the cDNA of jellyfish. This allowed us to overcome many of the difficulties associated with the previous method using fluorescent calcium indicaors [12]: apart from their difficulties in entering the cells, they tend to be sequestered into vesicles and give high autofluorescence. Furthermore, it requires dual excitation at 340 and 380 nm, which might cause radiation damage to the cells in the case of long exposures.

The results obtained in the present study showed that

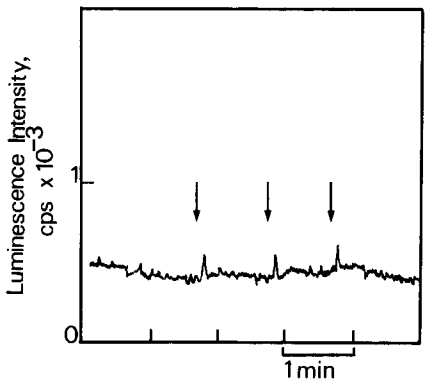

Fig. 3. Changes in $[(Ca^{2+})_i]$ in response to cAMP pulses by the aggregative cells in a calcium-deficient medium. Arrows indicate the points of addition of 50 nM cAMP.

the $[(Ca^{2+})_i]$ considerably increased (2.6-times) from the vegetative stage to the aggregation stage (Table 1), suggesting its involvement in cell adhesion and chemotactic cell movement required for the formation of multicellularity in development. As cells acquire responsiveness to exogenous cAMP signals during the pre-aggregation period, it was shown that they produce a small but significant transient increase in $[(Ca^{2+})_i]$ upon stimulation with cAMP (Fig. 3). Since these experiments were carried out in the complete absence of exogenous calcium, the transient increase must be due to release of sequestered calcium to the cytoplasm. This is supported by the recent finding of Flaadt et al. [14]. The following decrease in $[(Ca^{2+})_i]$ to the basal level is probably caused by re-sequesteration of cytosolic calcium. This transient increase was observed only when several cAMP pulses were given to the cells beforehand during the incubation with coelenterazine. This so-called 'priming' of the cells for cAMP stimulation has been shown to be necessary for synchronizing them for the stimulation. It is probable that repeated stimulation of aggregation-competent cells with autonomous cAMP signals is responsible for the marked increase in $[(Ca^{2+})_i]$ at the aggregation stage.

The cytosolic free calcium level was shown to be considerably decreased when migrating slugs were formed (Table 1). The results with separated prestalk and prespore cells (Table 2) indicate that the decrease is largely due to prespore cells, which consitute ca. 80% of a whole slug. The fact that the prestalk cells contain more $[(Ca^{2+})_i]$ than the prespore cells agrees with the previous result of Abe and Maeda [12] using fluorescent indicators. In spite of a low level of $[(Ca^{2+})_i]$ in slug cells, it showed a marked increase upon stimulation with cAMP (Table 1). This high responsiveness is limited only to prestalk cells (Fig. 4), as prespore cells exhibited a response as low as the aggregative cells. The result agrees with those of Abe and Maeda [12], although they used an unusually high concentration (10 μM) of cAMP for stimulation and the response was obscured due to large background signals. The marked difference in responsiveness of $[(Ca^{2+})_i]$ to cAMP stimulation between prestalk and prespore cells is consistent with the fact that

Table 2

Intracellular free calcium concentration, $[(Ca^{2+})_i]$, in prestalk and prespore cells and its increase upon stimulation with cAMP

Cell type	$[(Ca^{2+})_i]$ ± SD (nM)	
	Resting level	Increase upon cAMP (50 nM) stimulation
Prestalk	95 ± 5	138 ± 5.77
Prespore	41 ± 12.71	47 ± 21.44

All measurements were made at 10°C. n = 3–5.

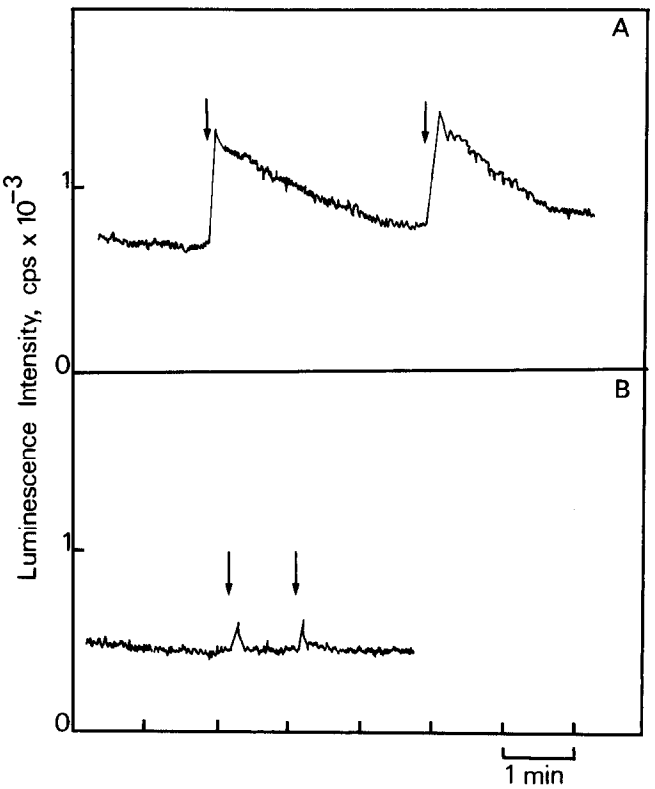

Fig. 4. Changes in $[(Ca^{2+})_i]$ in response to cAMP pulses by separated prestalk (A) and prespore (B) cells. Slug cells were separated by Percoll density gradient centrifugation, washed in a calcium-deficient medium and monitored for changes in $[(Ca^{2+})_i]$ (compare Fig. 3 with Fig. 4B). The arrows indicate the points of addition of 50 nM cAMP.

prestalk cells lead prespore cells in the morphogenetic movement during migration and culmination [20]. In fact, it has been shown that prestalk cells isolated from migrating slugs exhibit chemotaxis toward cAMP, whereas prespore cells do not [21]. Prestalk cells are also known to exert higher motive forces than prespore cells [22]. These facts are considered to be responsible for the separating out of prestalk and prespore cells which has been reported to occur when they are mixed [23,24]. The sorting out plays a key role in the formation of differentiation patterns in this organism [25,26].

The difference in $[(Ca^{2+})_i]$ and its response to cAMP stimulation between prestalk and prespore cells may be involved in differentiation of the two cell types. Involvement of calcium mobilization in post-aggregative differentiation was suggested by Schaap et al. [6] and Blumberg et al. [7] by the use of calcium channel blockers. However, the exact role of calcium in prestalk and prespore differentiation remains to be elucidated.

Transition from the migration stage to the culmination stage was shown to be associated with a large increase in both cytosolic free calcium levels and its response to cAMP stimulation, the latter showing the highest value during development (Table 1). This may be involved in the active ascending movement of the culminating cell

mass or in differentiation of mature stalk cells, as suggested by high concentrations of total [10] and sequestered calcium [11] in differentiating stalk cells.

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