# On the role of calcium during chemotactic signalling and differentiation of the cellular slime mould *Dictyostelium discoideum*

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**ABSTRACT Transient cytosolic calcium elevations are required for chemotaxis and differentiation of *Dictyostelium discoideum* since $Ca^{2+}$ chelating buffers introduced into the cells by scrape loading inhibited motility as well as orientation in a $Ca^{2+}$ specific manner. $Ca^{2+}$ changes are provided either by intrinsic cytosolic $Ca^{2+}$ oscillations that can be determined as periodic $Ca^{2+}$ efflux, or by receptor-mediated $Ca^{2+}$ liberation from the $InsP_3$-sensitive store and $Ca^{2+}$ influx. Cytosolic $Ca^{2+}$ homeostasis as well as oscillations seem to be regulated by two different $Ca^{2+}$ stores, the acidosomes and the $InsP_3$-sensitive store, both of which are dependent on $Ca^{2+}$ pumps and V-type $H^+$ ATPases. $Ca^{2+}$ transients are sensed by calmodulin-binding proteins. The latter have been detected in *Dictyostelium* by $^{125}$I-calmodulin labeling. A calmodulin-dependent protein phosphatase, calcineurin A, was cloned, sequenced, purified and characterized biochemically. Overproduction of calcineurin A as well as antisense constructs will help to the elucidation of its function in signal transduction. Surprisingly, protein synthesis is also controlled by $Ca^{2+}$/calmodulin. An integral ribosomal protein of the 60S subunit, L19, proved to be a calmodulin-binding protein and calmodulin antagonists of different classes, inhibited *in vitro* translation of *Dictyostelium* and wheat germ extracts.**

KEY WORDS: *cAMP, cGMP, oscillation, calcineurin, calmodulin*

## Introduction

The cellular slime mould serves as a model organism to study phagocytosis, chemotaxis, motility, bone-resorption, oscillations and differentiation (for review see Gerisch, 1987; Devreotes, 1989; Wurster *et al.*, 1990; Mutzel, 1991; Van Haastert, 1991).

Single motile cells feed on bacteria. Starvation induces a developmental program. cAMP is synthesized and released in a pulsatile manner from aggregation centers and attracts neighbouring cells to migrate to the center. cAMP binds to cell surface receptors (CAR1-4) and initiates a signalling cascade which includes transient increases of $InsP_3$, cGMP and finally cAMP. cAMP is relayed and attracts distantly located cells, thereby increasing the morphogenetic field of aggregating cells stepwise to 1-2 cm or 100,000 cells. The pulsatile release of cAMP results in concentric or spiral-rings of amoebae which finally collect in mounds. These mounds culminate or transform into a migrating slug which contains an organizing tip of prestalk cells and a larger rear end of prespore cells. This slug is sensitive to heat, light and humidity. After selection of a suitable place in the decaying foil a fruiting body is formed in a sort of gastrulation process whereby the tip invaginates forming a stalk and raises the spore mass. In the presence of bacteria single amoebae hatch from the spores.

We are interested in the mechanism of signal transduction following cAMP-binding, leading to chemotaxis, differentiation and oscillations. We found that cAMP causes a substantial amount of $Ca^{2+}$ influx and study the role of calcium in signal transduction.

## Calcium influx

$Ca^{2+}$ influx varies during differentiation. At the beginning influx is small, increases after 3 h and becomes maximal at 5 h. It starts 6-12 s after cAMP addition and peaks within 30 sec. It saturates at an extracellular calcium concentration of about 5-10 µM with a $K_{0.5}$ of 2 µM and yields a rate of influx of $3x10^8$ $Ca^{2+}$ ions per cell per min. This would result in an increase of $Ca_i$ of 10-15 µM if no $Ca^{2+}$ would be buffered or sequestered (Wick *et al.*, 1978; Bumann *et al.*, 1984).

There exist $2x10^8$ high affinity $Ca^{2+}$ binding sites per cell at the cell surface with a $K_{0.5}$ of 2 µM which are azide sensitive (Jaworski and Malchow, unpublished results), as is $Ca^{2+}$ influx (Böhme *et al.*, 1987).

*Abbreviations used in this paper:* CaM, calmodulin; cAR, cAMP receptor; $InsP_3$, inositol 1,4,5, triphosphate; PLC, phospholipase C.

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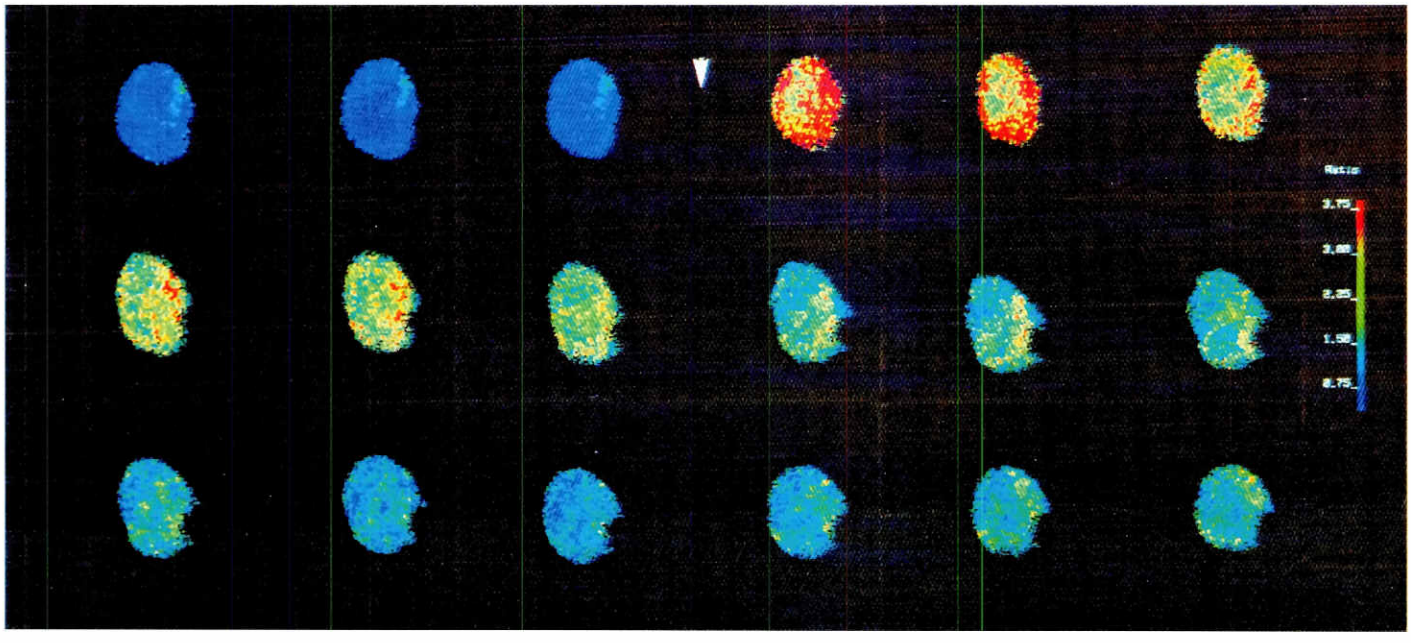

**Fig. 1. Calmidazolium induces a transient $Ca_i$ increase in aggregative *Dictyostelium* cells.** *15 µl 100 µM calmidazolium were added to 100 µl cells deposited on a slide, after scrape-loading with fura-2 (Schlatterer et al., 1992). The $Ca_i$ changes were recorded as described (Schlatterer et al., 1994) before (3 pictures) and after application of calmidazolium. The time point of addition is marked by an arrowhead. The pictures were taken 5 sec apart. The colour bar indicates the R 340/380 value which is proportional to the $Ca_i$ concentration. The external $Ca^{2+}$ concentration was 1 mM.*

Since $Ca^{2+}$ influx is measured as decrease of the extracellular $Ca^{2+}$ concentration with a $Ca^{2+}$ sensitive electrode or by association of $^{45}Ca^{2+}$ with the cells, $Ca^{2+}$ influx could reflect merely binding to the cells. However, receptor mediated influx is subject to inhibition by drugs that act on $Ca^{2+}$ transport ATPase or V-type $H^+$ ATPase (Flaadt *et al.*, 1993a), see below. Moreover, $Ca^{2+}$ influx is followed by $Ca^{2+}$ efflux. The amount of efflux is larger than influx during the beginning of differentiation. Later on, both are of equal size. During aggregation efflux gradually ceases and becomes totally absent. Under this condition $Ca^{2+}$ entry can be stimulated more than twenty times in the absence of $Ca^{2+}$ efflux. These results suggest that cAMP causes $Ca^{2+}$ entry into the cell and that $Ca^{2+}$ binding to the cell surface is a transitory event.

### *Mechanism of $Ca^{2+}$ influx*

The driving force for $Ca^{2+}$ entry is a $Ca^{2+}$ transport ATPase which sequesters $Ca^{2+}$ into an $InsP_3$-sensitive pool. A V-type $H^+$ ATPase seems to take part in this process similar to the situation in pancreatic acinar cells (Europe-Finner and Newell, 1986; Thevenod *et al.*, 1991; Flaadt *et al.*, 1993a).

Surprisingly, even in permeabilized cells, receptor-mediated $Ca^{2+}$ uptake was still in function and the $Ca^{2+}$ flux was blocked by inhibition of $Ca^{2+}$ transport ATPase or $H^+$ ATPase activity in permeabilized cells as well as in intact cells.

Our working hypothesis is that receptor-mediated activation of phospholipase C (PLC) yields $InsP_3$ which releases $Ca^{2+}$ from the $InsP_3$-sensitive pool. The empty store could elicit $Ca^{2+}$ entry at the plasma membrane and a feedback activation of $Ca^{2+}$ transport into the store. Concomitantly with $InsP_3$ formation cAMP activates membrane-bound guanylate cyclase activity. We have shown in streamer F mutants that display large aggregation territories due to a lack of cGMP phosphodiesterase, an enhanced and prolonged cAMP-induced $Ca^{2+}$ entry. We deduced that cGMP could be involved in opening of $Ca^{2+}$ channels similar as in visual transduction (Menz *et al.*, 1991). Indeed, a membrane-permeable cGMP-derivative activated the cAMP-induced $Ca^{2+}$ entry whereas cGMP which remains externally reduced the response due to receptor-desensitization (Flaadt *et*

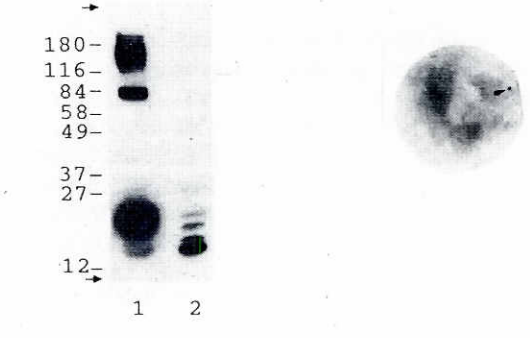


**Fig. 2. Identification and functional cloning of CaM-binding proteins from *Dictyostelium*.** *The left part of the figure shows binding of $^{125}I$-labeled D. discoideum CaM to total extract proteins from aggregative cells ($t_6$) blotted to a nitrocellulose membrane.* **(Lane 1)** *Binding in the presence of 0.1 mM $CaCl_2$;* **(lane 2)** *binding in the presence of 2 mM EGTA. The ca. 80 kDa band was identified as the Dictyostelium homolog of calcineurin, the 22 kDa band is the Dictyostelium CaM-binding homolog of mammalian ribosomal protein L19. The right part of the figure shows functional isolation of a cDNA clone for Dictyostelium calcineurin from a λgt11 lysogen expression library. In a background of 5000 recombinant clones a single colony expressing CaM-binding activity was identified (arrowhead). The cDNA insert from this clone encoded the C-terminal half of Dictyostelium calcineurin.*

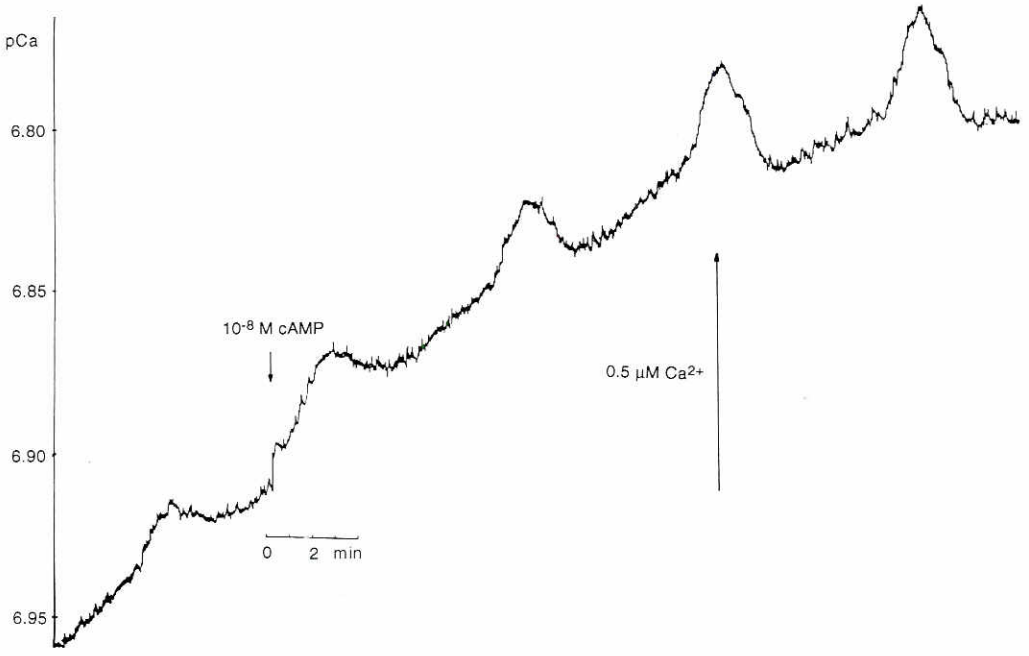


**Fig. 3. Extracellular $Ca^{2+}$ oscillations in mutant 21a.** *Note that oscillations begin with $Ca^{2+}$ efflux indicating that $Ca_i$ oscillates. A 10 nM cAMP pulse did not disturb the oscillations and induced a small amount of $Ca^{2+}$ release. The extracellular $Ca^{2+}$ concentration was recorded with a $Ca^{2+}$ sensitive electrode as described (Menz et al., 1991) in a suspension of $1x10^8$ cells per ml 5 h after induction of differentiation (Menz, 1988). The $Ca^{2+}$ efflux amounted to 30 pmol per $10^7$ cells.*

*al.*, 1993b). However, cGMP regulation is not a prerequisite for $Ca^{2+}$ entry, since the latter can occur in the absence of cGMP (Van Haastert, personal communication). cGMP seems to be used to increase $Ca^{2+}$ entry during spike formation (see below).

Likewise, Gα2 which is thought to activate PLC is not necessary for about half of the $Ca^{2+}$ flux (Milne and Devoreotes, 1993). Therefore, either $InsP_3$ can be formed in the absence of PLC activity which recently has been shown to occur by hydrolysis of $InsP_5$ (Drayer *et al.*, 1994) or a separate pathway of $Ca^{2+}$ entry must exist. Interestingly, even cells lacking Gα2 but possessing the full amount of cell surface receptor cAR1 (strain JM-1) sequester $Ca^{2+}$ into the $InsP_3$-sensitive store (unpublished).

Therefore, the PLC-independent formation of $InsP_3$ could be a major pathway in *Dictyostelium*. Alternatively, another messenger is involved in the regulation of the $InsP_3$-sensitive store.

### *Determination of intracellular calcium ($Ca_i$)*

To our surprise stimulation with low concentrations of cAMP did not cause an increase in $Ca_i$ as determined in single cells by scrape-loading of fura-2 (Schlatterer *et al.*, 1992). Measurement in cell suspension with aequorin expressing cells led to tiny short increases at low temperature (Saran *et al.*, 1994). An explanation to this discrepancy of the expected $Ca_i$ increase of 10-15 µM is that the cells rapidly sequester $Ca^{2+}$. This was demonstrated when cells were permeabilized with 3 mg/ml saponin in the presence of 1 mM external $Ca^{2+}$. Even after this harsh treatment the initial increase in $Ca_i$ was reduced to the resting level of the cytosolic $Ca^{2+}$ concentration (Schlatterer *et al.*, 1992).

Cytosolic $Ca_i$ increases were detected when high cAMP concentrations (100 µM) were applied (Schlatterer *et al.*, 1994). Under these conditions receptor-mediated $Ca^{2+}$ entry was independent of Gα2 and occurred even in vegetative cells. An increase of 50-100 nM over the basal level of 50 nM was found. Besides high cAMP concentrations calmidazolium induced an increase of $Ca_i$ in *Dictyostelium* (Fig. 1). The rise is preferentially due to $Ca^{2+}$ entry at the plasma membrane (Schlatterer, 1996).

Calcium sequestering organelles have been characterized by electron probe microanalysis (Schlatterer *et al.*, 1994). In addition to high amounts of $Ca^{2+}$ the mass dense granules contained phosphate and either $K^+$ or $Mg^{2+}$, depending on the state of differentiation.

Although the $Ca^{2+}$ concentration inside the cell is not increasing to a large extent, definite, possibly local $Ca^{2+}$ transients, do occur under physiological conditions. What then is the role of $Ca^{2+}$ elevations? At first we consider calmodulin-binding proteins.

## Calmodulin-binding proteins

One of the main primary receptors for elevated intracellular $Ca^{2+}$ in eukaryotic cells is calmodulin which, when complexed with $Ca^{2+}$, activates a number of target enzymes including protein kinases and protein phosphatases. In order to analyze these targets in *Dictyostelium* we are investigating, both at the biochemical and molecular level, proteins that bind to $Ca^{2+}$/CaM. Work from this and other laboratories has demonstrated the presence of a number of specific CaM-binding proteins in crude extracts and subcellular fractions (Winckler *et al.*, 1991). Probing protein blots from *Dictyostelium* cell extracts with $^{125}$I-labeled *Dictyostelium* CaM revealed several specific high-affinity CaM-binding proteins (Winckler *et al.*, 1991). In parallel, we have developed a novel technique for direct, functional isolation of cDNA clones for CaM-binding proteins by probing cDNA expression libraries with labeled CaM (Mutzel *et al.*, 1990; Mutzel, 1994). Using this approach, cDNA clones for a CaM-dependent protein phosphatase and for a ribosomal protein of the 60S subunit were isolated (Fig. 2).

### *Calcineurin*

$Ca^{2+}$/calmodulin-dependent protein phosphatase (calcineurin) plays a crucial role in the transduction of intracellular $Ca^{2+}$ signals to the gene level. In lymphocytes, the enzyme is a

major target of immunophilin-immunosuppressant complexes (Liu *et al.,* 1991) which inhibit its phosphatase activity. Concomitant with this inhibition are effects on the expression of interleukin genes. We have isolated and characterized a calcineurin homolog from *Dictyostelium* (Dammann *et al.,* 1996). There is evidence that calcineurin is part of a signaling cascade in *Dictyostelium* that regulates cell differentiation, similar to the situation in lymphocytes: *in vitro* differentiation studies by Gross and his co-workers (J. Gross, personal communication) show that cell-type proportions in *Dictyostelium* can be shifted by treatment both with calmodulin antagonists and the immunosuppressors, cyclosporin A and FK506. Recombinant *D. discoideum* cells that overexpress calcineurin (H. Dammann and R. Mutsel, unpublished) are analyzed, and we are trying to reduce the concentration of the protein by expression of antisense mRNA, and to disrupt the gene for calcineurin by homologous recombination. The reverse genetic approach is particularly promising in *Dictyostelium* since, unlike higher eukaryotes (or even yeast cells) this organism harbors only a single calcineurin gene. Since the *Dictyostelium* enzyme displays particular primary structure features as compared to its homologs from other eukaryotes (extra domains at both termini, a 4-fold repeated hexapeptide sequence immediately C-terminal from the putative autoinhibitory site) we are also investigating its biochemical properties. Recombinant protein was purified from *E. coli* cells and its biochemical properties determined (Damman *et al.,* 1996). We recently established a *Dictyostelium* cell line that over-expresses the protein about 50-fold, and we have developed a rapid and efficient purification strategy. The availability of pure *Dictyostelium* calcineurin A will now greatly facilitate its further biochemical and structural analysis.

### *Regulation of protein synthesis*

We have discovered a ribosomal $Ca^{2+}$/calmodulin-binding protein in *D. discoideum* that corresponds to the large ribosomal subunit protein L19 from mammalian cells and its homologs from archaebacteria (Sonnemann *et al.,* 1991). Hypothesizing that this protein could confer calmodulin regulation to the translation of mRNAs, we have initiated an investigation of the regulation of protein synthesis in *Dictyostelium*. An *in vitro* translation system has been established, and we find that elongation of nascent peptides in this system can indeed be specifically inhibited by calmodulin antagonists and monoclonal antibodies against *D. discoideum* calmodulin (Sonnemann *et al.,* 1993). These results are interesting in the light of recently published observations showing that in human *erbB-2* overexpressing tumor cells the mRNA for L19 is specifically overexpressed (Henry *et al.,* 1993). We are presently constructing *Dictyostelium* mutants with altered expression and primary structure of L19. *In vitro* translation assays using extracts from such cell lines will help understanding the role of L19 in protein synthesis and its regulation.

## Chemotaxis

Chemotactic movement is characterized by orientation and cell motility. To assess the question whether $Ca^{2+}$ transients are required for motility and/or orientation cells were loaded with $Ca^{2+}$ buffers of different $Ca^{2+}$ binding affinity (Schlatterer and Malchow, 1993; Unterweger and Schlatterer, 1995). It turned out that both motility and orientation depended on $Ca^{2+}$ elevations. Inhibition of orientation was less sensitive to extracellular $Ca^{2+}$ chelation than motility indicating that smaller $Ca^{2+}$ changes are sufficient for orientation to proceed. During pseudopod formation a gradient of $Ca_i$ increasing from the front to the rear was observed (Schlatterer *et al.,* 1994). In the streamer F mutants myosin II association with the cell cortex is enhanced and prolonged (Liu and Newell 1988). The same time dependence of wild-type and mutants was found for the rate of $Ca^{2+}$ influx, indicating that $Ca^{2+}$ plays a role in contraction (Menz *et al.,* 1991). Specifically we found an inhibition of phosphorylation of myosin II heavy chains by $Ca^{2+}$ (Malchow *et al.,* 1981) which was confirmed by Yumura and Kitanishi-Yumura (1993). Phosphorylation of myosin heavy chain results in removal of myosin from actin foci at the plasma membrane and subsequent dissociation of the myosin rods.

## Oscillations

As stated above cAMP is synthesized and released in a periodic manner. Light scattering measurements revealed the existence of spikes and sinus oscillations (Gerisch and Hess, 1974). Only the former are accompanied by large changes in cyclic nucleotide concentrations (Gerisch and Wick, 1975; Bumann *et al.,* 1986). There exists a basal (extracellular) $Ca^{2+}$ oscillation, which is transformed into spikes during signal relay (Bumann *et al.,* 1984, 1986). $Ca_i$ oscillations could not yet be detected, probably because of the efficient $Ca^{2+}$ sequestration. However, inhibitors of $Ca^{2+}$ transport ATPases or $H^+$ ATPases blocked light scattering spikes (unpublished). Besides $InsP_3$-sensitive $Ca^{2+}$ stores, acidosomes also seem to regulate $Ca_i$ in *Dictyostelium* (Rooney and Gross, 1992). Acidosomes are sensitive to the V-type $H^+$ ATPase inhibitor bafilomycin A1 whereas the $InsP_3$-sensitive pool is not; the latter is blocked by another V-type $H^+$ ATPase inhibitor, NBD-Cl (Flaadt *et al.,* 1993a). Since these compounds interfered transiently with spike formation, we infer that $Ca_i$ oscillations occur and that both $Ca^{2+}$ stores are intimately involved in spike generation. Indeed, in mutant cells selected for a strong chemotactic response, we measured periodic $Ca^{2+}$ release which was followed by influx (Fig. 3).

Since PLC is subject to feedback stimulation by $Ca_i$ (Lundberg and Newell, 1990; Cubitt and Firtel, 1992) and periodic changes of pH (Malchow *et al.,* 1978) as well as of potassium release (Aeckerle *et al.,* 1985) are occurring, which are also subject to control by $Ca^{2+}$ (Aeckerle and Malchow, 1989), a complex network seems to be responsible for generation of sustained $Ca^{2+}$ oscillations.

*Acknowledgments*

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