Biochemical and Biophysical Research Communications 425 (2012) 750–754

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Extracellular calmodulin regulates growth and cAMP-mediated chemotaxis in *Dictyostelium discoideum*

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ARTICLE INFO

Article history:
Received 22 July 2012
Available online 2 August 2012

Keywords:
Extracellular calmodulin
Eukaryotic microbe
Cell proliferation
Chemotaxis
Dictyostelium

ABSTRACT

The existence of extracellular calmodulin (CaM) has had a long and controversial history. CaM is a ubiquitous calcium-binding protein that has been found in every eukaryotic cell system. Calcium-free apo-CaM and Ca^{2+}/CaM exert their effects by binding to and regulating the activity of CaM-binding proteins (CaMBPs). Most of the research done to date on CaM and its CaMBPs has focused on their intracellular functions. The presence of extracellular CaM is well established in a number of plants where it functions in proliferation, cell wall regeneration, gene regulation and germination. While CaM has been detected extracellularly in several animal species, including frog, rat, rabbit and human, its extracellular localization and functions are less well established. In contrast the study of extracellular CaM in eukaryotic microbes remains to be done. Here we show that CaM is constitutively expressed and secreted throughout asexual development in *Dictyostelium* where the presence of extracellular CaM dose-dependently inhibits cell proliferation but increases cAMP mediated chemotaxis. During development, extracellular CaM localizes within the slime sheath where it coexists with at least one CaMBP, the matricellular CaM-binding protein CyrA. Coupled with previous research, this work provides direct evidence for the existence of extracellular CaM in the *Dictyostelium* and provides insight into its functions in this model amoebozoan.

1. Introduction

Calmodulin (CaM) is a ubiquitous Ca^{2+} binding protein common to eukaryotes [1–3]. While the intracellular localization and functions of CaM have been widely studied in plants, the presence of extracellular CaM (extCaM) in animals has been a somewhat controversial subject. In plants extCaM has been shown in a diversity of studies to regulate a number of different events. For example, extracellular CaM has been shown to affect cell division and cell wall regeneration as well as regulate subunit genes of Rubisco [4–6]. In addition, ExtCaM in plants has been shown to be involved in pollen germination, pollen tube growth and stomatal opening in *Arabidopsis* [7,8]. In animals the literature is less focussed but the accumulated data argues strongly that extCaM is a critical signaling element. For example, extCaM mediates nerve regeneration in frogs [9]. In hamsters, exogenous CaM amplifies peptide-mediated vasodilation [10]. Treatment with exogenous CaM stimulates DNA synthesis in rat liver cells [11]. In humans, extCaM appears to be required for cell division in the early preimplantation embryo [12] and increases proliferation of umbilical vein endothelial cells [13] and acts as a mitogen in keratinocytes [14]. These results suggest that extCaM acts as an autocrine growth factor. In contrast, the existence and function of extCaM in eukaryotic microbes has only recently been indicated.

In *Dictyostelium*, intracellular CaM (DdCaM; *calA*; DDB_G0279407) has been shown to have similar functions to CaM in other species [15,16]. A proteomic analysis of the extracellular medium from developing cells of *Dictyostelium* revealed the presence of (extCaM) as well as some putative calmodulin-binding proteins (CaMBPs) [17]. Subsequent research provided evidence that extCaM functions in the processing of the cysteine-rich, matricellular CaM-binding protein CyrA [18]. A subsequent study showed that immunoprecipitation of matricellular CyrA also pulled down CaM indicating that these proteins interact extracellularly [19].

Here we provide additional evidence showing that extracellular CaM exists and functions throughout growth and development of *Dictyostelium*. During the growth phase extCaM acts to inhibit cell proliferation but later stimulates cAMP chemotaxis as cells transit to asexual development. As cells continue development, extCaM

Abbreviations: extCaM, extracellular CaM; DdCaM, *Dictyostelium* calmodulin.

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http://dx.doi.org/10.1016/j.bbrc.2012.07.147

localizes to the extracellular matrix (ECM) surrounding the multicellular migratory pseudoplasmodium where it associates with the CaMBP CyrA. Thus in *Dictyostelium*, extCaM exists and serves several functions supporting the idea that extracellular CaM may be just as ubiquitous evolutionarily as intracellular CaM.

2. Materials & methods

2.1. Growth, chemotaxis and developmental analysis

Dictyostelium discoideum strain AX3 was grown either in the presence of *Escherichia coli* on SM agar or axenically in HL-5 medium as described previously [19,20]. cAMP-mediated chemotaxis assays were performed as previously described [21–23]. Cells for the chemotaxis assays were starved for 2 h in KK2 buffer and shaken at 22 °C and 120 rpm before plating on 0.5% agar containing cAMP (10 μM) and the indicated concentration of CaM or BSA. Means and standard errors were calculated for controls and experimental conditions and statistical analyses were conducted using the Mann–Whitney two-sample rank U-test. For development AX3 cells were washed in development buffer (DB; 5 mM Na_2H-PO4, 5 mM KH_2PO_4, 1 mM $CaCl_2$, 2 mM $MgCl_2$), plated on filters and harvested, as previously described [19,20].

2.2. Slime sheath isolation

Multicellular slugs were produced by plating drops of vegetative *Dictyostelium* strain WS380B cells, originally grown with *E. coli* on SM agar, onto 2% KK2 agar, and kept in complete darkness. During this time, slugs were allowed to migrate towards a pin-sized light source. The wild-type WS380B strain (DBS0235798; http://www.dictybase.org) was selected because of its ability to form slugs that are able to migrate for long periods of time. Immunohistochemistry of slugs and the slime sheath was performed as detailed previously [18]. Slime sheath was removed from the surface of 5–10 agar plates with KK2 buffer and a cell spreader. The harvested sheath was lyophilized, resuspended in 1 ml of 2% SDS, and incubated at 30 °C for 2 h. β-mercaptoethanol was added to a final concentration of 1% followed by sample loading buffer to a final concentration of 1×. The solution was boiled for 5 min. Samples were stored at −80 °C for future use. Aliquots of harvested slime sheath were separated by SDS–PAGE and analyzed by western blotting.

2.3. Asexual development and intracellular expression analysis

Protein expression during asexual development was assayed as previously described [18,19]. Developing AX3 cells were maintained on filters in a humidity chamber at 22 °C and samples were collected every 4 h using cold DB. Harvested cells were lysed in NP-40 lysis buffer containing 0.5% NP-40, 50 mM Tris–HCl pH 8.0, 150 mM sodium chloride, 1 μg/ml pepstatin A, 5 mM EDTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and a protease inhibitor cocktail tablet (Hoffmann-La Roche Limited, Mississauga, ON, Canada). For extracellular samples, the DB supernatant was cleared of cells by centrifugation (verified with a haemocytometer) and concentrated at least 40 times using an Amicon Ultra Centrifugal Filter Device-15 with a molecular weight cut-off of 10 kDa (Millipore Corporation, Billerica, MA, USA). All samples were stored at -80 °C for future use.

2.4. SDS–PAGE and Western blotting

SDS–PAGE and western blotting were performed as previously described [18,19,24]. The following primary antibodies were used: rabbit polyclonal anti-C-CyrA [1:1000; Suarez et al., 2011]; mouse monoclonal anti-tubulin (1:1000; Developmental Studies Hybridoma Bank, University of Iowa, IA, USA); and mouse monoclonal anti-CaM (6D4; 1:1000; EMD Biosciences Inc., La Jolla, CA, USA). Membranes were developed with the Amersham™ ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) and scanned using a Storm™ 860 Phosphorimager/Fluorimager (Molecular Dynamics Inc., Sunnyvale, CA, USA).

3. Results & discussion

3.1. DdCaM expression during growth and development

Early studies on DdCaM (*calA*; DDB_G0279407) gene expression showed that its mRNA is constitutively expressed throughout

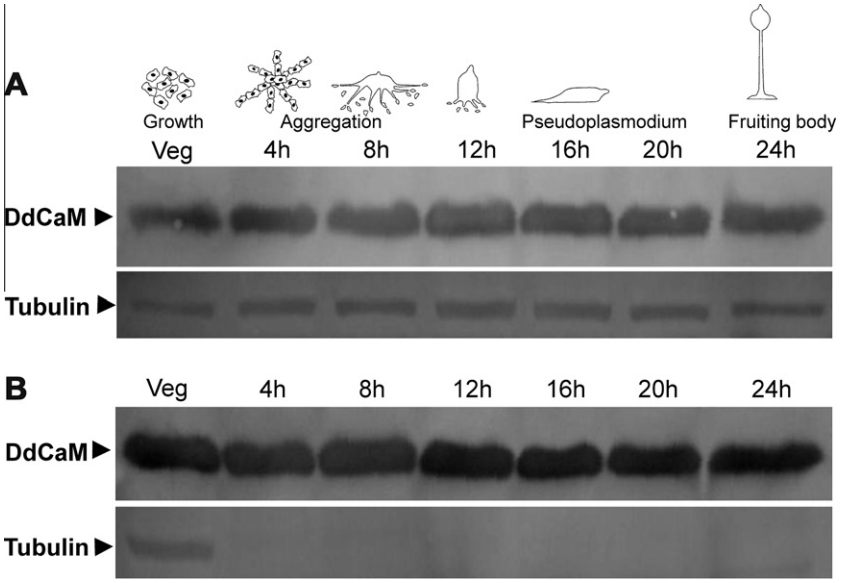

Fig. 1. Intracellular and extracellular *Dictyostelium* CaM protein levels throughout development. *Dictyostelium* AX3 was starved in DB and allowed to develop on filters. Samples were harvested at the desired developmental stages and separated into cellular (A) and extracellular fractions (B) and processed for western blotting. The Veg lane in both blots represents whole cell lysate and extracellular medium from vegetative cultures. Blots were probed with anti-CaM or anti-tubulin.

development [15; http://dictyexpress.biolab.si]. Since mRNA expression levels are not always indicative of protein expression, the intracellular and extracellular expression levels of DdCaM protein were examined. The expression of intracellular DdCaM coincided with mRNA expression indicating the protein is also constitutively expressed during development (Fig. 1A). Based on proteome analysis [19], the presence of extracellular CaM during growth was expected (Fig. 1B, Veg). Unexpectedly however were the high and constant levels of extracellular DdCaM that were present during starvation, through aggregation (cAMP mediated-chemotaxis), pseudoplasmodium formation and culmination (Fig. 1B). The extracellular accumulation of extCaM was not due to cell lysis as verified by the lack of tubulin protein (Fig. 1B) as well as the lack of visual or quantitative evidence of cell death or lysis (data not shown).

3.2. Exogenous CaM inhibits cell proliferation

The presence of extCaM in vegetative cells shown here and detected in proteome data by others [17] suggested it might be involved in regulating the growth of *Dictyostelium* amoebae. As seen in Fig. 3A, treatment of axenic cells with 10 μg/ml of bovine CaM did not affect proliferation. However a marked decrease in the rate of cell proliferation occurred at 50 μg/ml. At 100 μg/ml bovine CaM essentially stopped growth in axenic cultures. Bovine CaM (89% sequence identity with DdCaM) has previously been shown to be functionally equivalent to DdCaM [25]. Adding bovine serum albumin (BSA) as a control did not affect axenic growth (Fig. 2B). While some studies have shown that extracellular CaM can increase cell proliferation, other studies have shown that the presence increased levels of extCaM can inhibit proliferation while the outgrowth of sensory axons and the proliferation of non-neuronal cells in frog also were shown to be inhibited by exogenous CaM [9,11–14].

3.3. Exogenous CaM enhances cAMP-mediated chemotaxis

Since extCaM was present during aggregation, which involves cAMP-mediated chemotaxis, it raised the possibility that it functioned during this event. Our lab has previously shown that antagonizing DdCaM inhibits cAMP-mediated chemotaxis [21]. In keeping with this, treatment with 50 and 100 μg/ml bovine CaM significantly enhanced cAMP-mediated chemotaxis, while 10 μg/ml was ineffective (Fig. 4). In contrast, BSA treatment did not affect cAMP-mediated chemotaxis (Fig. 4).

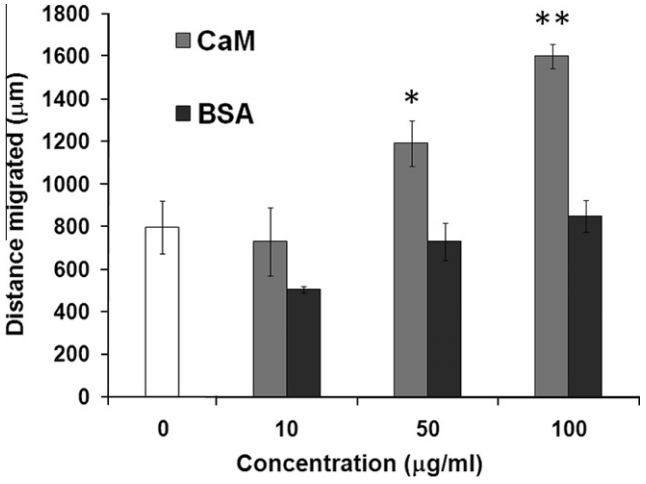

Fig. 3. Effect of exogenous CaM and BSA on cAMP-mediated chemotaxis. Cells were starved for 2 h in KK_2 phosphate buffer prior to plating on 0.5% agar containing 10 μM cAMP ± CaM or BSA. Data presented as the mean distance migrated ± SEM ($n = 4$). Mann–Whitney two-sample rank U-test ($^{*}p = 0.01$ vs. 0 μg/ml; $^{**}p = 0.005$ vs. 0 μg/ml).

3.4. Extracellular CaM in the ECM of Dictyostelium

As development continues, the multicellular tissue-like aggregate continues to grow upward as cells enter causing it to rise up from the substratum. Ultimately it will either culminate directly or fall over as a multicellular pseudoplasmodium or slug. The slug is highly motile and covered in an ECM that forms at its tip forming a tube of proteins, glycoproteins and carbohydrates through which the cells move [26]. As slug movement progresses, the so called "slime sheath" is left behind as a trail of ECM that can be harvested and analyzed. The work presented here verifies that extCaM is present at this stage and previous work has shown DdCaM binds to the cysteine-rich, matricellular CaMBP CyrA that accumulates within the ECM [18]. As a result we examined the localization of extCaM within the slime sheath via immunostaining. Immunolocalization of CaM shows that it is present homogeneously through the ECM at the front of slugs (Fig. 4A left panel, B) but begins to cluster into CaM-rich deposits in the middle of the slug (Fig. 4A center panel, B). Near the rear of the slug almost all of the extCaM

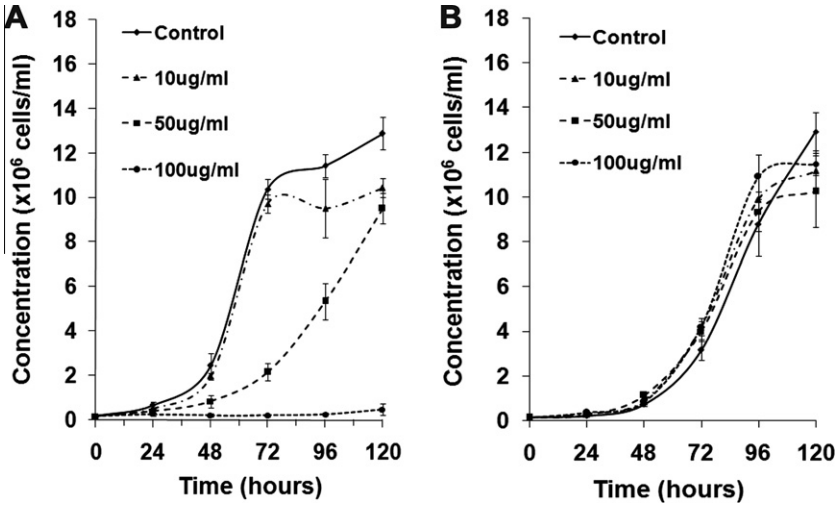

Fig. 2. Effect of exogenous (A) CaM and (B) BSA on axenic growth. AX3 cells were grown in HL-5 media ± CaM or BSA. The concentration of both control and CaM- or BSA-treated cultures was measured every 24 h over a 120 h period. Data presented as mean concentration + SEM ($n = 4–5$).

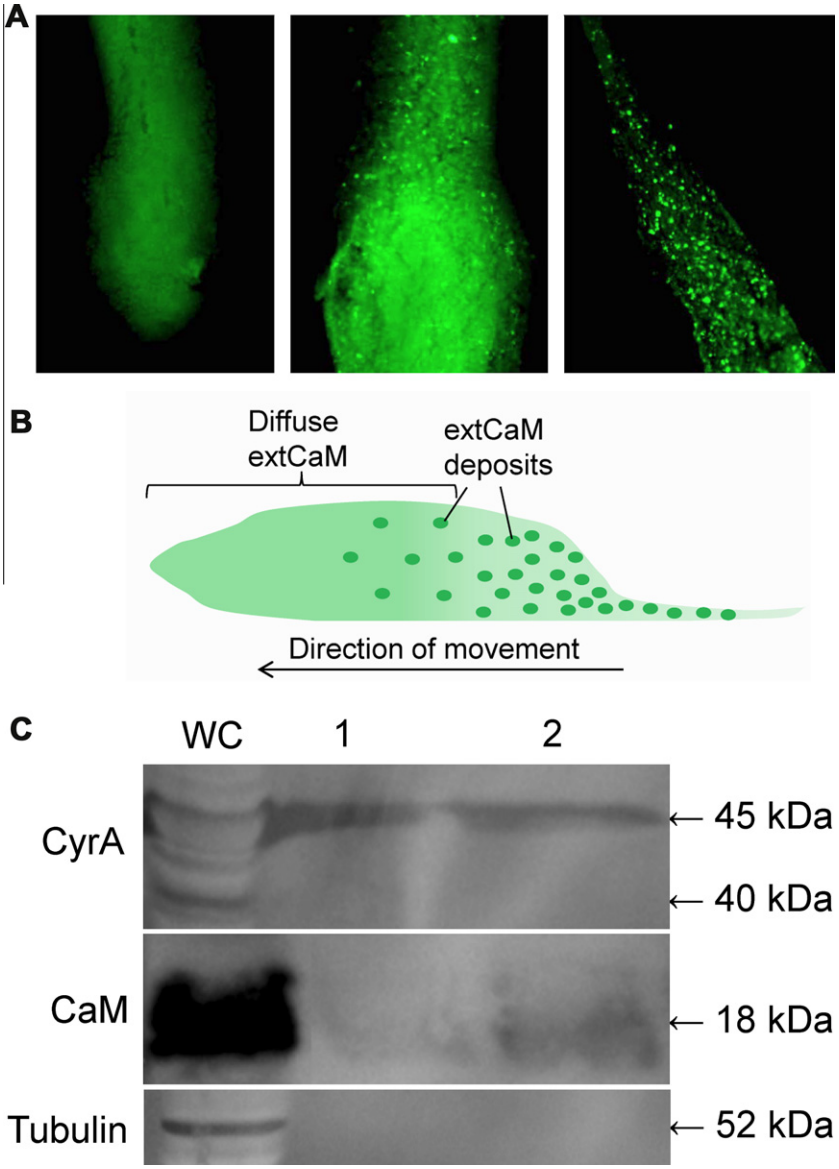

Fig. 4. Detection of CyrA and CaM in the slime sheath. (A) Immunolocalization of DdCaM in AX3 pseudoplasmodium anterior (left panel), middle (center) and posterior sections (right). (B) Illustration of extCaM localization in pseudoplasmodium. (C) Presence of CyrA and CaM in isolated slime sheath deposited by WS380B slugs as shown via western blotting. Western blots probed with anti-C-CyrA, anti-CaM, or anti-tubulin.

is in these punctate accumulations (Fig. 4A right panel, B). Staining for cells shows that they are not present within these deposits (data not shown). In addition, when the sheath is purified and subjected to western blotting, CaM is clearly present while the cell marker protein tubulin is notably absent (Fig. 4B). The presence of CyrA, a cysteine-rich, matricellular, CaMBP was also detected in the slime sheath analyzed in this study. Previously, CaM was shown to protect extracellular CyrA from proteolysis [18]. Since CaM exerts its effects by binding to and regulating CaMBPs, the presence of extracellular targets, such as CyrA, are a critical element in validating the external functions of CaM.

The localization of extCaM in the pseudoplasmodium is intriguing and may be important to the regulation of CaM during multicellular development. The transition in localization from uniform CaM distribution at the tip of the slug, through a combination of uniform and punctate localization in the middle to almost solely punctate in the rear is interesting. The CaMBP CyrA binds to CaM and at least one EGF-like domain present within CyrA has been shown to increase cAMP-mediated chemotaxis [22,23,27]. This data suggests that CaM is free at the tip and front of the slug where the highest levels of cAMP are present which mediate chemotaxis of slug cells [28]. Thus the free CaM could enhance the motility of cells at the front of the slug. Farther and farther back, the levels of extCaM and hence its role in augmenting chemotaxis would be diminished by its concentration in CaM deposits and thus its apparent loss as soluble extCaM. The movement of the pseudoplasmodium is a complex process that remains to be fully elucidated. It is well established that calcium and calmodulin mediate cAMP chemotaxis and the data presented here suggest that extCaM may be one additional signaling component that is involved in slug movement and morphogenesis.

The presence of extracellular CaM presents a problem for the indiscriminate use of calmodulin antagonists. Clearly such pharmacological interventions would benefit from co-experiments utilizing both cell-impermeable and permeable antagonists to differentiate between the intracellular and extracellular functions of CaM. This and other aspects of the regulation, function and translocation of extCaM remain to be studied.

These and previous results reveal that extracellular CaM is present in the eukaryotic amoebozoan *Dictyostelium* where it mediates at least three functions: proteolytic processing of CyrA, cell proliferation, cAMP mediated chemotaxis and possibly pseudoplasmodium motility and morphogenesis. Together with the work by others on plants and animals, this suggests that extracellular CaM may be just as ubiquitous evolutionarily and as functionally critical as intracellular CaM.

Acknowledgments

This work was supported by a Grant (D.H.O'D.; A6807) and Canada Graduate Scholarship (R.J.H.) from the Natural Sciences and Engineering Research Council of Canada.

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