The in vivo role of annexin VII (synexin): characterization of an annexin VII-deficient Dictyostelium mutant indicates an involvement in Ca\(^{2+}\)-regulated processes

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Summary

Dictyostelium discoideum cells harbor two annexin VII isoforms of 47 and 51 kDa which are present throughout development. In immunofluorescence and cell fractionation studies annexin VII was found in the cytoplasm and on the plasma membrane. In gene disruption mutants lacking both annexin VII isoforms growth, pinocytosis, phagocytosis, chemotaxis and motility were not significantly impaired under routine laboratory conditions, and the cells were able to complete the developmental cycle on bacterial plates. On non-nutrient agar plates development was delayed by three to four hours and a significant number of aggregates was no longer able to form fruiting bodies. Exocytosis as determined by measuring extracellular cAMP phosphodiesterase, \(\alpha\)-fucosidase and \(\alpha\)-mannosidase activity was unaltered, the total amounts of these enzymes were however lower in the mutant than in the wild type. The mutant cells were markedly impaired when they were exposed to low Ca\(^{2+}\) concentrations by adding EGTA to the nutrient medium. Under these conditions growth, motility and chemotaxis were severely affected. The Ca\(^{2+}\) concentrations were similar in mutant and wild-type cells both under normal and Ca\(^{2+}\) limiting conditions; however, the distribution was altered under low Ca\(^{2+}\) conditions in SYN\(^-\) cells. The data suggest that annexin VII is not required for membrane fusion events but rather contributes to proper Ca\(^{2+}\) homeostasis in the cell.

Key words: annexin, calcium, endocytosis, exocytosis, motility

Introduction

Annexins are eukaryotic Ca\(^{2+}\) binding proteins with common biochemical as well as structural characteristics. They exhibit Ca\(^{2+}\)-dependent binding to natural and artificial phospholipid membranes (Klee, 1988). The amino acid sequences reveal a dipartite structure composed of a conserved core domain and a variable N terminus. The core domain consists of a 4 or 8 times imperfectly repeated segment of about 70 amino acids. Each segment forms a compact mainly \(\alpha\)-helical subdomain as shown by 3-dimensional crystal structure analysis for annexin V (Huber et al., 1990a). According to this structure the four annexin core repeats surround a central pore forming a putative Ca\(^{2+}\) channel. Multiple Ca\(^{2+}\) binding sites have been detected which are all located on one face of the molecule that is supposed to be the membrane attachment side (Huber et al., 1990b; Demange et al., 1994). The N-terminal domains of the annexins are usually short, consisting of less than 60 amino acids. Their amino acid composition diverges; on the basis of these differences the annexins have been classified meanwhile into 13 different species (Crompton et al., 1988; Crompton and Dedman, 1990).

Annexins have been initially identified in mammalian cells but are also present in phylogenetically distant organisms like Hydra (Schlaepfer et al., 1992) and Dictyostelium discoideum (Döring et al., 1991; Gerke, 1991; Greenwood and Tsang, 1991). Despite a wealth of structural and biochemical data the physiological role of the annexins is still not known. A number of functions have been proposed which are based mainly on in vitro data and are related to the Ca\(^{2+}\)/phospholipid binding properties of annexins. Some activities appear to be confined to higher organisms, including phospholipase A2 inhibition (Pepinsky et al., 1986, 1988) and inhibition of blood coagulation (Tait et al., 1988). However, they can be explained well by Ca\(^{2+}\)-dependent sequestration of phospholipids by the annexins. More general cellular aspects in which annexins are believed to be involved are cytoskeleton-membrane interactions (Gerke and Weber, 1984; Massey et al., 1991), ion-anneling (Pollard and Rojas, 1988; Pollard et al., 1990; Rojas et al., 1990) and membrane traffic (Drust and Creutz, 1988; Ali
et al., 1989; Sarafian et al., 1991; Lin et al., 1992; Emans et al., 1993). In the latter case, an involvement of annexin VI in endocytosis as seen for fibroblasts (Lin et al., 1992) appears not to be a general phenomenon, since it was not confirmed for human A431 cells (Smythe et al., 1994).

Annexin (annexin VII) is unique among the annexins in having an unusually long N-terminal domain of more than 100 amino acids which is particularly rich in glycine, proline and tyrosine residues. Until now, cDNAs from human, mouse and Dictyostelium have been cloned and sequenced (Burns et al., 1989; Döring et al., 1991; Zhang-Keck et al., 1993). In addition to its remarkable sequence the synxin N terminus is subject to polymorphisms. On the mRNA and protein level two isoforms of human and mouse synxin have been identified which differ in the length of their N-terminal tails (Magendzo et al., 1989; Selbert et al., 1995). Most probably they arise by differential splicing. In Dictyostelium annexin VII the situation seems to be similar. The gene contains a cassette exon coding which differs in the length of their N-terminal tails (Magendzo et al., 1989; Selbert et al., 1995). Most probably they arise by differential splicing. In addition, the intracellular distribution of annexin VII may create an exocytotic pore at the site of vesicle/plasma membrane fusion. In addition, annexin VII could be involved in maintaining the Ca$^{2+}$-homeostasis within the cell. This is particularly tempting, since the selectivity of the channel for Ca$^{2+}$ is very high (Pollard and Rojas, 1988). However, a relation between the activities of annexin VII and its physiological function remains to be established.

To elucidate the role of annexin VII a Dictyostelium mutant lacking annexin VII mRNA and protein was obtained by gene inactivation via homologous recombination (Döring et al., 1991). Preliminary studies showed no significant defects in the mutant’s ability to grow or develop in association with bacteria. In this report the characterization of the mutant focussed on aspects of membrane trafficking and Ca$^{2+}$ regulation. In addition, the intracellular distribution of annexin VII in D. discoideum was determined and the expression of annexin VII during development investigated.

**MATERIALS AND METHODS**

**Growth and development of Dictyostelium cells**

D. discoideum strain AX2 and transformants were grown at 21°C in liquid nutrient medium at 160 rpm (Claviez et al., 1982) or on SM agar plates with Klebsiella aerogenes (Noegel et al., 1985). The annexin VII-minus cells have been described previously (Döring et al., 1991). Transformant B that lacks a functional annexin VII was renamed SYN-. Transformants were cultivated in liquid nutrient medium in the presence of 20 mg/ml genetin (Sigma Corp., Deisenhofen, FRG). For analysis of development cells were grown axenically to a density of 1 to 2×10⁶ cells/ml, washed with Soerensen phosphate buffer, pH 6.0, and deposited on phosphate agar plates (Witke et al., 1992; Faix et al., 1992) or nitrocellulose filters (Noegel et al., 1985), or resuspended in Soerensen phosphate buffer at a density of 1×10⁶ cells per ml and kept in shaken suspension for the indicated time period. Geneticin was no longer present during starvation. For calcium depletion, cells were grown in liquid nutrient medium in the presence of various concentrations of EGTA. The pH was carefully checked and found not to be affected by the addition of EGTA. Development of Ca$^{2+}$-depleted cells for determination of enzyme secretion, motility and chemotaxis analysis was in Soerensen phosphate buffer, pH 6.0, which is essentially Ca$^{2+}$-free.

**Subcellular fractionation**

Subcellular fractionation was done as described (Weiner et al., 1993). Briefly, cells were harvested, washed in Soerensen phosphate buffer, pH 6.0, resuspended in 0.25 M sucrose-TKM (50 mM Tris-HCl, pH 7.6, 25 mM KCl, 5 mM MgCl₂, 5 mM DTT; Ebert et al., 1989) containing a mixture of protease inhibitors and lysed in a Parr bomb by nitrogen cavitation after equilibration for 10 minutes at 1,000 psi or by passage through Nucleopore filters (PC-MEMB, 47 mm, SOUM, OTY 100, Costar, Cambridge, MA). Unbroken cells and nuclei were removed by sequential centrifugation at 450 g for 2 minutes and 700 g for 5 minutes. At this stage Ca$^{2+}$ was added to the supernatant as indicated. To separate membranes from cytoplasm, the supernatant was fractionated on a sucrose step gradient of 0.25 and 2 M sucrose in TKM. Centrifugation was for 1 hour at 180,000 g. Membranes were further separated on discontinuous sucrose gradients (Hohmann et al., 1985; Cardelli et al., 1989). The distribution of membranes and organelles in the gradients was assessed by measuring the distribution of alkaline phosphatase as plasma membrane marker (Loomis, 1969), acid phosphatase as lysosomal marker (Loomis and Kuspa, 1984) and comitin as Golgi marker (Weiner et al., 1993). Using this fractionation scheme, Golgi and plasma membranes partition in the same region of the gradient. For analysis of annexin VII and comitin distribution, 0.33 volume % of each fraction were separated by SDS-PAGE followed by immunoblotting.

**Production of monoclonal antibodies and immunofluorescence labelling**

Balb/c mice were immunized with bacterially expressed protein. For recombinant protein expression, a 1,400 bp EcoRIV/HindIII fragment comprising the complete core domain of annexin VII (Döring et al., 1991) was cloned into the Escherichia coli expression vector pMSS5 (Simon et al., 1988). The 35 kDa protein could be extracted from inclusion bodies with 4 M urea. Fusions were performed 3 days after the last boost, essentially as described (Schleicher et al., 1984) using 63Ag8-653 and PAIB3Ag8I myeloma lines. Hybridoma supernatants were screened for their ability to recognize annexin VII in D. discoideum cellular extracts. The antibodies designated 185-312-1, 185-338-1 and 186-447-1 were used in this study. For immunofluorescence D. discoideum growth phase cells were seeded on coverslips and allowed to attach for 20 minutes. Cells were fixed with methanol for 10 minutes at −20°C, washed twice for 5 minutes with PBS, twice for 15 minutes with PBG (0.5% BSA, 0.1% gelatine in PBS) and incubated for 2 to 12 hours with anti-annexin VII antibody (mAb 185-312-1 or 185-338-1). The cells were then washed five times with PBG and incubated for one hour with 1:200 diluted FITC-conjugated, affinity-purified goat anti-mouse IgG (Cappel, Cochranville, PA). After washing with PBG (three times) and PBS (three times), the cells were embedded in Gelvatol (Langanger et al., 1983) and examined with a Zeiss Axiophot fluorescence microscope. The agar overlay method was used as described (Fukui et al., 1987), and the cells were fixed in methanol. For control, specimens were incubated with the secondary antibody only. Specificity of the antibodies in immunofluorescence was also checked with mutant cells lacking annexin VII, which were essentially devoid of staining.
Phagocytosis and pinocytosis assays

Phagocytosis of fluorescent latex beads was assayed as described by Witke et al. (1992). Pinocytosis was measured with [3H]dextran as fluid-phase marker (Vogel, 1987). D. discoideum cells were grown to a density of 1 to 2×10⁶ cells, washed twice with 17 mM Soerensen phosphate buffer, pH 6.0, resuspended in axenic medium at a density of 4×10⁶ cells/ml and kept shaking at 160 rpm at 21°C for 30 minutes. The [3H]dextran (>3.7 GBq/g) (Amersham, Braunschweig, FRG) was added at a concentration of 1 µCi/ml. At the indicated time points aliquots of 1 ml were removed in triplicate, the cells pelleted, washed twice with axenic medium, lysed in 100 µl 0.5% Triton X-100 (Roth, Karlsruhe, FRG) and the radioactivity was measured in a scintillation counter (Packard, Canberra, Australia). The experiments were carried out two times independently.

Measurement of Ca²⁺ uptake and release

Ca²⁺ uptake and release were measured using ⁴⁵Ca²⁺ (>370 GBq/g) (NEN, Dreieich, FRG) according to Milne and Coukell (1991). For determination of uptake, cells were washed twice in H-buffer (20 mM Hepes/KOH, pH 7.0, 5 mM KCl) and resuspended at a density of 5×10⁶ cells/ml in H-buffer. The cells were kept shaking at 160 rpm at 21°C. For analysis of uptake 100 µl of cell suspension were removed at the indicated time points and added to 100 µl of uptake buffer (20 mM Hepes/KOH, pH 7.0, 5 mM KCl, 50 µM CaCl₂, 0.5 µCi ⁴⁵CaCl₂). The samples were vigorously mixed and the ⁴⁵Ca²⁺ uptake stopped by adding H-buffer containing 775 mM CaCl₂. The cells were pelleted, washed in H-buffer containing 10 mM CaCl₂, lysed in 2% SDS and the radioactivity was determined in a scintillation counter.

For measurement of Ca²⁺ release 2 ml of uptake buffer were added to a cell suspension in H-buffer (5×10⁷ cells/ml). Shaking was continued for one minute, EGTA (5 mM final concn) was added and at distinct time points 400 µl aliquots were removed. The cells were pelleted, washed and lysed as described above and cell-associated radioactivity was determined. To measure folate-induced Ca²⁺ uptake or release, 40 µM of folate was added and samples were taken after various time points. Measurements of internal Ca²⁺ concentrations were done as described (Schlatterer et al., 1992, 1994).

Motility and chemotaxis assays

To assay chemotaxis, cells after 6 hours of starvation were transferred onto a glass surface, stimulated with micropipettes filled with 1 mM cAMP as described (Gerisch et al., 1975a,b) and examined in a Zeiss inverted microscope equipped with phase-contrast optics. In order to obtain quantitative data on motility and chemotaxis, cells were analyzed in a chamber using an image processing system as described by Segall et al. (1987) and Fisher et al. (1989). When Ca²⁺-depleted cells were assayed, EGTA was also present during motion analysis.

Miscellaneous methods

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970), and immunoblotting using the method of Towbin et al. (1979). Protein was determined according to the method of Lowry et al. (1951) or Bradford (1976). Phosphodiesterase activity was determined according to Gerisch et al. (1972), α-mannosidase activity according to Dimond et al. (1983), α-fucosidase activity according to Schopohl et al. (1992). All experiments were repeated three to five times. DNA and RNA were analyzed according to André et al. (1988).

RESULTS

Dictyostelium contains two isoforms of annexin VII

To obtain monoclonal antibodies specific for annexin VII, the bacterially expressed core domain was used as an immunogen, thereby avoiding possible cross-reactivity with other GYPPQ-containing proteins previously identified in Dictyostelium (Noegel et al., 1990). In whole cell homogenates the monoclonal antibodies recognized two proteins of 47 and 51 kDa, which were both present throughout development (Fig. 1). Since annexin VII is encoded in D. discoideum by a single gene (Döring et al., 1991) the two proteins are most likely produced by alternative splicing of a cassette exon coding for 43 amino acids, which extend the N-terminal part (Greenwood and Tsang, 1991). The 47 kDa protein species corresponds in size to the product of a cDNA previously described (Döring et al., 1991) and turned out to be the major annexin VII variant in all developmental stages. For the large isoform an increase in amount was noted at the onset of development (Fig. 1).

Localization of annexin VII in D. discoideum cells

Annexin VII-specific antibodies were used for immunofluorescence labelling of fixed D. discoideum cells. The plasma membrane was most strongly labelled, with pseudopodial extrusions being excluded, but label was also found throughout the cytoplasm, indicating that there are a membrane-bound and a cytosolic form of the protein (Fig. 2A and B). Using the agar-overlay technique to stain D. discoideum cells, additional label became visible at the cell nucleus (Fig. 2A). The presence of annexin VII in nuclei has also been reported for mammalian cells (Kuijpers et al., 1993). In cell fractionation experiments in the presence of 100 µM Ca²⁺ annexin VII was almost entirely associated with membranes (Fig. 3A). Further fractionation of these membranes on a discontinuous sucrose gradient showed the protein to be present in fractions containing plasma membrane and Golgi and to a lesser extent also in fractions containing endosomes and lysosomes (Fig 3B). In the absence of Ca²⁺ almost all annexin VII was in the soluble fraction.

Growth and development of an annexin VII-minus mutant (SYN-)

A D. discoideum mutant lacking annexin VII mRNA was generated by homologous recombination (Döring et al., 1991). Annexin VII-specific antibodies confirmed the complete absence of both the 47 and 51 kDa isoforms in the SYN- mutant. The growth curve of the SYN- cells in axenic medium showed an extended lag phase after inoculation at low cell density (Fig. 4). After the titer had reached about 1×10⁵ cells/ml, cells entered the logarithmic growth phase and

![Fig. 1. Presence of annexin VII during Dictyostelium development. Total cellular extracts from D. discoideum strain AX2 (2×10⁵ cells per lane) were separated by SDS-PAGE (12% acrylamide). An immunoblot was prepared using directly iodinated mAb 185-338-1. Development was on nitrocellulose filters: 12 hours after the beginning of starvation corresponds to aggregation stage; 15 hours to culmination; and after 18 hours fruiting bodies are formed. The molecular masses of the annexin VII isoforms are indicated.](Image)
continued to grow at an almost normal rate. Growth on a lawn of bacteria was not affected.

*D. discoideum* starts a developmental program when deprived of nutrients. During a 24 hour period the cells form aggregates which undergo morphological changes before culminating into a fruiting body. SYN<sup>−</sup> cells were able to complete the developmental cycle when cultivated on SM-agar with *K. aerogenes*. When deposited on phosphate agar plates or on nitrocellulose filters, more than half of the aggregates did not develop into fruiting bodies (Fig. 5). Furthermore, under these conditions cells entered development 3 to 4 hours later than wild-type AX2 as revealed with stage-specific markers (data not shown).

**Transport processes in SYN<sup>−</sup> cells**

In phagocytosis assays the mutant showed a normal rate of incorporation of fluorescent 1 µm beads during a 20 minute period (Fig. 6A). The ability of the mutant to pinocytose [<sup>3</sup>H]dextran, a fluid-phase marker, was also not significantly different from the control (Fig. 6B). As in wild-type cells, an almost linear accumulation during the first hour was followed by a steady decrease in the uptake rate until 5 hours of incubation.

To examine exocytosis, the secretion of lysosomal hydrolases and of cAMP phosphodiesterase was assayed. Since the phosphodiesterase is induced by cAMP (Malchow et al., 1972),
Annexin VII-deficient Dictyostelium mutant

its release was tested with cells incubated in 1 mM cAMP during the first 3 hours of development. For both mutant and wild-type cells a linear increase in extracellular PDE activity was found with no difference within the limits of 10 to 15% (Fig. 7).

The expression of lysosomal α-mannosidase occurs during growth and the first hours of development (Schatzle et al., 1991). Regulation is at the transcriptional level. The enzyme undergoes a variety of posttranslational modifications during its transit from the cytosol via ER and Golgi to the late lysosomes. There the final maturation steps involving proteolytic processing take place. The mature enzyme is secreted in a regulated fashion during growth phase and development, most likely via vesicles which shuttle between lysosomes and the plasma membrane (Cardelli et al., 1990). The mutant cells were tested for their ability to secrete α-mannosidase during the first hours of development; 70% of the total enzyme activity was found in the medium of mutant and wild-type cells after 3 hours of development in shaking suspension (Fig. 8). After 6 hours a plateau was reached with about 80% secreted enzyme. Although secretion occurred normally in the mutant, there was consistently less active enzyme present in SYN− cells, amounting to only 64% of wild-type activity. Secretion of lysosomal enzymes can be stimulated by sugars that are not metabolized (Crean and Rossomando, 1979). The presence of 100 mM sucrose in the starvation medium led to an increase
in the rate as well as in the extent of secretion of α-mannosidase; 90% of the active enzyme was found in the medium (Fig. 8B). Again, the SYN− cells did not show a deficiency in their secretory capacity.

Determination of α-fucosidase activity, a lysosomal enzyme that is released from cells during early development, showed that the SYN− cells secreted the enzyme in a fashion comparable to wild-type cells (data not shown). The total enzyme activity was lowered in the mutant by 31%.

**Effect of low Ca²⁺ concentrations on growth**

Annexin VII has been shown to act as a voltage-gated Ca²⁺-selective channel in artificial as well as in natural phospholipid bilayers (Pollard and Rojas, 1988; Pollard et al., 1990). This finding implies a role for the protein in regulating the Ca²⁺ flux between different intracellular compartments or between the cytoplasm and the external space. Absence of annexin VII might therefore affect the calcium homeostasis in the cell. A change in the intracellular Ca²⁺ concentration can be induced by the addition of the Ca²⁺ chelator EGTA to the growth medium. EGTA traps the calcium which is constantly leaching out of the cells (Wick et al., 1978). The tolerance of SYN− cells towards depletion of the intracellular Ca²⁺ content was tested by growing them in the presence of different EGTA concentrations in axenic medium. Growth of the mutant cells was severely impaired by 1 mM, and almost completely inhibited by 3 mM EGTA. Growth of wild-type cells was not affected in the presence of 1 mM EGTA, and 3 mM EGTA had only a small effect (Fig. 9). Survival of cells grown in the presence of EGTA was assayed by plating them onto SM agar. This...
showed that the non-dividing mutant cells were still viable after 14 hours in a suspension containing 3 mM EGTA. After 20 to 30 hours the survival rate declined. Addition of excess Ca²⁺ to EGTA-containing medium restored growth to normal.

**Exocytosis under low Ca²⁺ concentrations**

Exocytosis was assayed with cells kept during growth in the presence of 3 mM EGTA for 13 to 14 hours. To account for the reduced division rate of the mutant under these experimental conditions, mutant cell cultures had been inoculated with a higher cell number. In wild-type and mutant cells EGTA treatment reduced the amount of secreted α-mannosidase in a comparable fashion. In untreated cells 70% of the total activity was secreted, in Ca²⁺-depleted cells 55% was secreted. However, in the mutant the total α-mannosidase activity was reduced to 42% compared to similarly treated wild-type cells (Fig. 10). Extracellular phosphodiesterase (PDE) levels were affected as well. For AX2 cells a reduction of approximately 30% compared to untreated cells, and in the SYN⁻ cells a reduction of approximately 50%, was observed (data not shown). Northern blot analysis showed that the reduction in extracellular PDE was paralleled by a decrease in PDE-specific RNA, which could be mediated by an effect of Ca²⁺ on gene expression as observed previously (Preston et al., 1990). The main effect of lowered Ca²⁺ levels therefore appears to be on synthesis of secreted enzymes rather than on exocytosis.

**Cell motility and chemotaxis**

Ca²⁺ is thought to be a key regulator of cell motility. Chemotaxis towards the chemoattractant cAMP and motility of the SYN⁻ mutant were similar to those in the wild type when cells were grown under routine conditions and assayed after 6 hours of starvation. Ca²⁺ deprivation by growth in the presence of 3 mM EGTA led to a general reduction in the speed of 50%. Whereas wild-type cells were able to orient themselves in a cAMP gradient, orientation of mutant cells towards cAMP and motility was heavily impaired (Table 1). Lower concentrations of EGTA did not affect orientation and motility as strongly, and at 0.5 mM EGTA chemotaxis and motility were almost completely restored (data not shown).

**Ca²⁺ uptake and release**

The movement of Ca²⁺ between cytoplasm and medium can be tested by measuring the uptake and release of ⁴⁵Ca²⁺. A
slow basal uptake of \(^{45}\text{Ca}^{2+}\) which is due to an exchange of the intracellular \(\text{Ca}^{2+}\) content can be distinguished from a transient rise in the \(^{45}\text{Ca}^{2+}\) influx upon addition of the chemoattractants folate and cAMP to vegetative and aggregation competent cells, respectively (Wick et al., 1978; Milne and Coukell, 1991). The basal and folate-induced \(^{45}\text{Ca}^{2+}\) uptake of axenically grown SYN\(^{-}\) and wild-type cells was measured. The kinetics of \(^{45}\text{Ca}^{2+}\) influx exhibited by the mutant deviated only slightly from that of the wild type (Fig. 11A). Both cell lines showed a fast increase in their intracellular \(^{45}\text{Ca}^{2+}\) content at 5 to 6 seconds after addition of 40 \(\mu\text{M}\) folate to the cell suspension. After about 40 seconds a plateau of increase was reached. The plateau value of the mutant cells, as well as the basal uptake rate of the unstimulated cells, was slightly lower than those observed for the control.

Release of calcium was measured from cells which were loaded with \(^{45}\text{Ca}^{2+}\) for 1 minute after a folate stimulus. Addition of 5 mM EGTA to cell suspensions led to a rapid \(^{45}\text{Ca}^{2+}\) efflux during the first 3 to 4 minutes (Fig. 11B). After 10 minutes 80 to 85% of the loaded \(^{45}\text{Ca}^{2+}\) was found in the extracellular medium. The efflux rates of mutant and wild type were comparable. The intracellular \(\text{Ca}^{2+}\) concentrations of mutant and wild-type cells as determined using photometric measurements did not differ significantly from each other. Pretreatment with EGTA led to a reduction of intracellular \(\text{Ca}^{2+}\) in both cells to extremely low levels. Under these conditions in SYN\(^{-}\) cells the residual \(\text{Ca}^{2+}\) was strongly enriched in vesicles which were not observed in wild-type cells (Fig. 11C).

### Table 1. Motility parameters of AX2 and SYN\(^{-}\) cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Speed ((\mu\text{m/min}))</th>
<th>Orientation ((\cos \theta))</th>
<th>Turns</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Without cAMP gradient</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AX2</td>
<td>4.8±1.3</td>
<td>−0.06±0.03</td>
<td>50±11</td>
<td>6</td>
</tr>
<tr>
<td>SYN(^{-})</td>
<td>4.8±1.6</td>
<td>−0.08±0.17</td>
<td>53±5</td>
<td>6</td>
</tr>
<tr>
<td>AX2 (EGTA)</td>
<td>2.0±0.7</td>
<td>−0.13±0.15</td>
<td>45±11</td>
<td>7</td>
</tr>
<tr>
<td>SYN(^{-}) (EGTA)</td>
<td>2.3±0.5</td>
<td>0.03±0.03</td>
<td>53±9</td>
<td>4</td>
</tr>
<tr>
<td><strong>With cAMP gradient</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AX2</td>
<td>10.8±1.6</td>
<td>0.26±0.15</td>
<td>42±4</td>
<td>6</td>
</tr>
<tr>
<td>SYN(^{-})</td>
<td>11.1±2.0</td>
<td>0.29±0.13</td>
<td>44±5</td>
<td>6</td>
</tr>
<tr>
<td>AX2 (EGTA)</td>
<td>6.3±1.9</td>
<td>0.29±0.13</td>
<td>42±6</td>
<td>7</td>
</tr>
<tr>
<td>SYN(^{-}) (EGTA)</td>
<td>2.3±0.5</td>
<td>0.03±0.03</td>
<td>53±9</td>
<td>4</td>
</tr>
</tbody>
</table>

Cells grown in axenic medium in the presence or absence of 3 mM EGTA as indicated were starved for six hours and deposited on a glass surface coated with BSA, which was placed into a chemotaxis chamber. Speed of cell movement and orientation were recorded for 30 minutes, a cAMP gradient was established and recording continued. The determination of the motility parameters was done according to Fisher et al. (1989) and Segall et al. (1987). Orientation is the fraction of the distance travelled per time-lapse interval that is in the direction of the gradient. Turns (rad²/min) indicate the change in the direction of movement. The data are averages of the indicated numbers of experiments (n).

### DISCUSSION

Annexin VII (synexin) was originally isolated in an attempt to identify the proteins catalyzing the contact of the secretory vesicle membranes and their fusion in vitro. The presence of annexin VII in cells that normally do not exhibit a specialized secretory system suggested that it could represent a constitutive element of membrane trafficking as well. A role in membrane trafficking has been suggested for annexins in general and two recent reports specifically pointed out an involvement of annexin II and annexin VI in endocytosis (Emans et al., 1993; Lin et al., 1992). In *D. discoideum* the localization of annexin VII on the plasma membrane and in the cytosol would be compatible with a role in membrane trafficking. However, membrane binding of annexin VII is only detected when the cells are opened in the presence of 100 \(\mu\text{M}\) \(\text{Ca}^{2+}\). This finding indicates that local \(\text{Ca}^{2+}\) concentrations are
Fig. 10. Effect of EGTA on \(\alpha\)-mannosidase secretion. The \(\alpha\)-mannosidase activity of cells after growth for 13 to 14 hours in the presence of 3 mM EGTA was determined at the indicated hours of starvation. In (A) the total activity of the mutant is shown as compared to total wild-type activity. Open circles: comparison of the activities after growth in axenic medium. Filled circles: comparison of the activities after growth in axenic medium supplemented with 3 mM EGTA. (B) Secreted \(\alpha\)-mannosidase after growth in axenic medium with 3 mM EGTA. Values are from one representative experiment.

Fig. 11. \(\text{Ca}^{2+}\) uptake and release. (A) Basal (triangles) and folate-stimulated (circles) \(\text{Ca}^{2+}\) uptake: 0.5 \(\mu\text{Ci} \ ^{45}\text{Ca}^{2+}\) was added to 100 \(\mu\text{l}\) of cells (5 \(\times\) 10⁷/ml) and cell-associated radioactivity was determined. Uptake was stimulated by the addition of 40 \(\mu\text{M}\) folate. (B) For determination of \(\text{Ca}^{2+}\) release cells were allowed to accumulate \(^{45}\text{Ca}^{2+}\). The decrease of cell-associated radioactivity was followed after addition of 5 mM EGTA. The results shown are the mean values of 3 to 5 experiments. (C) Photometric determination of \(\text{Ca}^{2+}\) distribution in AX2 wild-type (a) and SYN⁻ cells (b). Cells were grown in the presence of 3 mM EGTA. Loading of cells with fura-2 and measurements were done according to Schlatterer et al. (1992).
important for membrane association of the protein, which could then play a regulatory role. An investigation of the secretory capacity of SYN− cells for several extracellular enzymes showed that secretion was not impaired but that the total enzyme activity was consistently lower. Coukell et al. (1992) had observed a similar defect for phosphodiesterase and its inhibitor when Dicystostelium cells were grown in the presence of agents that affected intracellular calcium levels. They showed that the reduced levels were due to degradation of the proteins in the endoplasmic reticulum (ER) and concluded that low intracellular calcium disrupted normal functions of the secretory pathway. Nearly unaltered phagocytosis and pinocytosis by the Dicystostelium SYN− mutant, as well as the capacity to secrete extracellular enzymes, do not argue for a stringent role of annexin VII in exo- and endocytic processes, the reduced enzyme activities could however be due to an involvement in the secretory pathway. In analogy to the data on phosphodiesterase (Milne and Coukell, 1991) the annexin VII-dependent inhibition of secretion would not lead to a feed-back accumulation of secretory enzymes inside the cell but rather to an increased degradation in the ER, resulting in an overall reduced enzyme activity. Alternatively, reduced enzyme activities could be due to altered transcription levels of the respective mRNAs. Reduced levels of PDE-specific mRNA were in fact observed for SYN− cells after growth in either the presence or absence of EGTA. This could be caused by either a reduced transcription rate or altered posttranscriptional regulation. For prolactin gene expression, which shows a Ca2+-specific enhancement, effects of Ca2+ at the transcriptional and the posttranscriptional level were demonstrated (Preston et al., 1990). The Ca2+-mediated increase in prolactin mRNA levels by posttranscriptional mechanisms was found to be due to posttranslational modification of a stable protein. Similarly, the reduced α-mannosidase and fuscosidase activities could also result from altered transcript levels.

The involvement of annexin VII in transcription could be a direct one, since staining of nuclei with annexin VII antibodies has been observed, or annexin VII could affect regulatory mechanisms via its interaction with Ca2+. This latter view appears even more likely in the context of results obtained with Ca2+-depleted SYN− cells, which showed an impairment in growth, motility and chemotaxis.

In the laboratory cells are kept under optimal growth conditions and normally do not experience dramatic alterations. This could obscure a potential role for annexin VII that is related to calcium. Ca2+ is thought to allow the aggregation of phospholipid layers by neutralizing surface charges. Annexin VII supposedly acts in a synergistic manner and a lack of annexin VII could be overcome by increased calcium concentrations. If this were the case, lowered calcium levels should no longer be tolerated by the SYN− cells. The addition of EGTA as an efficient Ca2+-chelator had a marked effect on growth of SYN− cells and inhibited cell division at a concentration of 3 mM strongly, whereas wild-type cells were nearly not affected. Ca2+ depletion also affected the levels of extracellular enzymes. This was not so much due to altering the rate of secretion but rather to a further reduction in total enzyme activities, pointing to participation of annexin VII in the regulation of the Ca2+ levels in cells. The failure of the SYN− cells to undergo normal development on phosphate agar, which is essentially Ca2+-free, would also support such a role. Ca2+ has been previously shown to be important for development (Mason et al., 1971; Marin and Rothman, 1980).

Depletion of extracellular Ca2+ affects the motile behavior of cells, and Van Duijn and Van Haastert (1992) showed that Ca2+ is required for motility but not for orientation in a chemotactic gradient and pseudopod formation. Under normal laboratory conditions Syn− and control cells exhibited comparable motility and chemotaxis. After Ca2+ depletion wild-type cells were still motile although the speed was significantly reduced. Chemotaxis was not impaired and the cells showed normal responses to cAMP. In the Syn− cells EGTA treatment resulted similarly in a reduction of speed, but after addition of cAMP the cells were not able to orientate in the gradient. Reducing the EGTA concentrations led to an improvement of motility and chemotactic behavior. Van Duijn and Van Haastert (1992) had suggested previously that for locomotion of cells a global increase in Ca2+ is required whereas for pseudopod formation local Ca2+ changes are necessary, which might still occur under the experimental conditions they used but are no longer possible in the SYN− cells.

Support for this comes from determination of the overall Ca2+ levels in mutant and wild type, which did not differ significantly from each other either under control conditions or at low Ca2+ concentration. However, under the latter conditions the distribution of the residual Ca2+ appeared to be altered and it could very well be that this Ca2+ is no longer available for cellular reactions. The precise role of annexin VII in this process has yet to be elucidated.

In summary, essential cell functions like phagocytosis, pinocytosis and secretion of extracellular enzymes are not drastically altered in the SYN− mutant. This suggests that annexin VII does not play a key role in these pathways. However, as soon as Ca2+ levels are reduced, the SYN− cells, in contrast to wild-type cells, are severely impaired in growth, production of extracellular enzymes, development, motility and chemotaxis, which are all processes that are influenced by changing the Ca2+ concentration.

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