The Response of *Physarum polycephalum* to Extracellular Ca^{2+} : Studies on Ca^{2+} Nutrition, Ca^{2+} Fluxes and Ca^{2+} Compartmentation

BY ROSS P. HOLMES* AND PETER R. STEWART

Department of Biochemistry, Faculty of Science, Australian National University,
Canberra, ACT 2600, Australia

(Received 6 November 1978)

The calcium requirement for optimal growth of *Physarum polycephalum* is between 0.05 and 2.5 mM. Media without Ca^{2+} will not support growth though Ca^{2+} -chelating agents, such as EDTA and EGTA, do not have any marked effect on Ca^{2+} availability at the low pH used to culture this organism. The intracellular Ca^{2+} concentration varies approximately 10-fold when the external Ca^{2+} concentration is varied between 0.05 and 25 mM. Ca^{2+} is concentrated in the mitochondrial and microsomal fractions, but the high concentration found in the cytosol suggests that considerable redistribution of Ca^{2+} occurs during disruption and fractionation of the plasmodia. $^{45}\text{Ca}^{2+}$ uptake by intact microplasmodia occurs at rates which are comparable to those required for Ca^{2+} uptake during growth and generation of new cell mass. This uptake process has two components: one non-saturable with Ca^{2+} , and the other saturable with an apparent maximum velocity of $67 \text{ nmol } \text{Ca}^{2+} \text{ h}^{-1} (\text{mg protein})^{-1}$ and an apparent K_m of 1.9 mM-Ca^{2+} . As judged by $[^3\text{H}]\text{inulin}$ uptake, part of the $^{45}\text{Ca}^{2+}$ uptake may be endocytotic.

Ionophores A23187 and Br-X537A stimulate $^{45}\text{Ca}^{2+}$ uptake. Uptake is inhibited by KCl, Mn^{2+} and Sr^{2+} at high concentrations, but is not sensitive to La^{3+} at concentrations similar to Ca^{2+} in the uptake medium. KCN and iodoacetic acid inhibit the transport, while cytochalasin B, verapamil, tetracaine and procaine have little or no effect.

INTRODUCTION

Calcium ions have been implicated in the regulation of many metabolic processes. These include cell adhesion, cell-cell communication, cell division, membrane structure and permeability, secretion, transmission of nerve impulses, fluxes of metabolites through metabolic pathways and actomyosin-based contractile processes (Borle, 1973; Rasmussen, 1975; Berridge, 1975). There is, however, only limited information available concerning the quantitative requirement of cells for Ca^{2+} and the mechanisms by which Ca^{2+} enters cells. Clearly, Ca^{2+} should be an essential requirement for the growth of all cells, though an absolute requirement for Ca^{2+} has sometimes been difficult to demonstrate. Balk *et al.* (1973), for example, found that transformed fibroblasts could proliferate in medium in which the free Ca^{2+} concentration approached zero [achieved by chelation with 1,2-di(2-aminoethoxy)ethane-*N,N,N',N'*-tetraacetic acid (EGTA)], whereas untransformed cells could not proliferate in this medium.

The slime mould *Physarum polycephalum* is being used increasingly in studies of the role of Ca^{2+} regulation of motility in eukaryotic cells (see, for example, Hatano, 1970; Ridgway

* Present address: Burnside Research Laboratory, University of Illinois, Urbana, Illinois 61801, U.S.A.

& Durham, 1976; Holmes & Stewart, 1977). The extent to which internal cellular compartments respond to variation in external Ca^{2+} concentration is important to an understanding of the contribution and relationship of the different compartments and their associated transport systems in regulating the intracellular Ca^{2+} concentration.

In this report, we describe experiments on the growth requirement of *P. polycephalum* for Ca^{2+} , the influx and efflux of $^{45}\text{Ca}^{2+}$ into and out of plasmodia, the effects of potential inhibitors of Ca^{2+} uptake, and the Ca^{2+} content of subcellular compartments. The relationship of these experiments to possible mechanisms of Ca^{2+} movement across the plasma membrane is discussed.

METHODS

Culture conditions. *Physarum polycephalum* M₃C was from Dr K. Babcock, of the MacArdle Laboratory for Cancer Research, University of Wisconsin, Madison, U.S.A. In growth experiments where the Ca^{2+} content of the medium was varied, the semi-defined medium (CSD) recommended by Carlile (1971) was used except that EDTA was omitted. Stock cultures were maintained on modified Carlile medium (MCM), in which the biotin and thiamin of CSD were replaced by 0.5% (w/v) yeast extract (Difco). In initial experiments the medium (PRM) of Daniel & Baldwin (1964) was used. Both MCM and PRM were equally effective in supporting growth with a generation time of approximately 9 h at 25°C. The defined medium (DM) of Dee *et al.* (1973) was used in experiments in which rates and extent of growth were measured. Macroplasmodia were grown on glass beads as outlined by Guttus & Guttus (1964).

Growth experiments. The rate of growth was determined by assaying the pigment (Daniel & Baldwin, 1964) and protein content of microplasmodial pellets obtained by centrifuging samples of the culture at 250 g for 1 min. Pellets were washed once in 5 ml 20 mM-citric acid/NaOH buffer (pH 4.6) containing 2.5 mM- CaCl_2 , then resuspended in 4% (w/v) trichloroacetic acid in 50% (v/v) acetone and left at room temperature for 30 min. After centrifuging at 1500 g for 5 min, the pigment content was read as absorbance at 400 nm and protein was assayed in the pellet after dissolving it in 1 M-NaOH. Protein and pigment increased in parallel during growth of microplasmodia except when 25 mM- Ca^{2+} was present; under the latter conditions, pigment increased about 30% slower than protein.

Ca^{2+} analysis of subcellular fractions. Microplasmodial pellets, washed in citrate buffer without CaCl_2 , were homogenized in 10 vol. ice-cold 0.25 M-sucrose/10 mM-N-2-hydroxyethylpiperazine ethanesulphonic acid (HEPES; pH 7.4)/1 mM-EGTA, using 10 strokes in an all-glass Dounce homogenizer (B pestle, 15 ml capacity). A low-speed pellet, which consisted of aggregated membrane fragments and some nuclei, was collected by centrifuging at 500 g for 2 min. A mitochondrial fraction was obtained by subsequent centrifuging at 3000 g for 6 min (Holmes & Stewart, 1979) and a membrane vesicle fraction by centrifuging the post-mitochondrial supernatant at 20000 g for 15 min. Centrifuging the post-vesicle supernatant at 160000 g for 90 min yielded a microsomal pellet (Kato & Tonomura, 1977). The mitochondrial and vesicle fractions were washed once with homogenizing medium. Samples of fractions were assayed for protein by Lowry's method.

The Ca^{2+} content of plasmodia and subcellular fractions was measured as follows. Samples (0.5 to 1.0 ml) were ashed with 1 ml 2.5 M- H_2SO_4 /1 M- HClO_4 at 160 to 170°C in an oil bath for 45 min. The ashed material was then oxidized by adding a drop of H_2O_2 at 30 min intervals until oxidation (as evidenced by the disappearance of carbon) was complete. Samples were diluted with KCl solution to 50 mM- K^+ . The Ca^{2+} content was assayed using an air/nitrous oxide/acetylene flame in a Varian Techtron model 1200 atomic absorption spectrophotometer. Buffer blanks were digested and assayed in the same way and used to correct sample assays.

Ca^{2+} uptake. Ca^{2+} uptake was measured in 40 ml of exponential phase culture in a 250 ml flask shaken in a water bath at 25°C. At intervals after adding $^{45}\text{Ca}^{2+}$ (0.2 $\mu\text{Ci ml}^{-1}$), 2.0 ml samples were removed and rapidly filtered through 2.5 cm diam. Gelman Type E glass-fibre discs presoaked in wash medium. Filters were washed with 10 ml of growth medium which included either a 10-fold excess of unlabelled Ca^{2+} , or 20 mM- MgCl_2 /100 mM-KCl, to prevent binding of $^{45}\text{Ca}^{2+}$ to low affinity sites on the cell surface. Filters were dried and the radioactivity was determined by liquid scintillation counting. To obtain meaningful and reproducible uptake it was essential to use cultures in which microplasmodia were small. This was achieved by growing microplasmodia in CSD without added Ca^{2+} and then transferring them to MCM (with Ca^{2+} at the required concentration) 2 d before cultures were needed.

Ca^{2+} efflux. Microplasmodia were grown in MCM for 5 to 6 generations (2 d) with $^{45}\text{Ca}^{2+}$ (2 $\mu\text{Ci ml}^{-1}$). Cultures (50 ml) were harvested and washed quickly in 50 ml of unlabelled medium, resuspended in 50 ml of fresh unlabelled medium and shaken in a 250 ml flask at 25°C. At intervals, 3 ml samples were taken, rapidly filtered through glass-fibre discs, and the $^{45}\text{Ca}^{2+}$ in the filtrate was estimated.

Fig. 1. Effect of culture dilution on growth rate. Cultures were grown in PRM; growth was measured by pigment formation, expressed as the absorbance at 400 nm of a 5.0 ml acid/acetone extract of 1 ml culture (see Methods). At the time indicated by the arrow, the culture (○) was diluted 1/3 (●), 1/10 (□) or 1/30 (■). The bars show the period of slow growth rate after dilution.

Materials. Chelex-100 was obtained from Bio-Rad Laboratories, A23187 was a gift from Lilly Research Laboratories, Indianapolis, U.S.A., and Br-X537A was from Roche Products, Sydney, Australia. A23187 was dissolved in dimethyl sulphoxide and Br-X537A in methanol (both at 10 mM) and stored at -20°C .

RESULTS

Growth experiments

Increased lag phases resulted when exponential phase cultures were inoculated into fresh medium to give increasing dilutions (Fig. 1). To avoid this effect, low dilutions of stock cultures (generally 1/5 to 1/10) were made in growth experiments. Dilution in 'old' medium, i.e. medium conditioned by prior growth of microplasmodial cultures in it, overcame the dilution effect. The possible nature of the medium-conditioning factor was not examined further.

Effect of medium Ca²⁺ concentration on growth

The relationship between growth rate, measured by the rate of protein and pigment increase, and the Ca²⁺ concentration in both defined and semi-defined media is shown in Table 1. Both CSD and DM contain significant amounts of Ca²⁺ (50 and 20 μM , respectively) contributed by various components of the media. In semi-defined medium, microplasmodia tolerated a wide range of Ca²⁺ concentrations without any adverse effect on the growth rate; however, the growth rate was decreased with 25 mM-Ca²⁺, and both the growth rate and yield were decreased when the Ca²⁺ concentration was 50 μM or lower. In defined medium, the growth rate and yield were unimpaired when 50 μM -Ca²⁺ was added. Without added Ca²⁺, there was an extensive lag period before limited growth occurred. The subsequent growth rate was not affected but the yield was only 20% of that with added Ca²⁺. In CSD prepared with peptone and glucose that had been passed through a Chelex-100 column to remove contaminating divalent cations, no growth was detected. When 1 mM-CaCl₂ was added to this medium, the growth rate and yield obtained were similar to those with untreated glucose and peptone.

In both semi-defined and defined media without added Ca²⁺, microplasmodia were much

Table 1. *Effect of Ca²⁺ and chelators on growth of microplasmodia*

Microplasmodia were cultured in liquid media as described in Methods. Duplicate samples were removed at intervals, microplasmodia were pelleted, and the protein contents of the pellets were determined. The exponential growth rate was calculated from the slope of the growth curve during exponential growth. Yield was determined at stationary phase.

Medium*	Ca ²⁺ concn† (mM)	Exponential growth rate (generations h ⁻¹)	Yield (mg protein ml ⁻¹)
CSD	25.05	0.050	2.1
CSD	2.55	0.091	2.1
CSD	0.30	0.091	2.0
CSD	0.05	0.056	1.2
CSD	0	0	0
DM	1.02	0.023	0.9
DM	0.07	0.024	0.9
DM	0.02	0.023	0.2
CSD + 0.67 mM-EDTA	0.05	0.054	1.5
CSD + 0.5 mM-EGTA	0.05	0.045	1.3
DM + 0.5 mM-EGTA	0.07	0.010	0.4

* CSD, Semi-defined medium (Carlile, 1971); DM, defined medium (Dee *et al.*, 1973).

† Basal CSD contained 50 μ M Ca²⁺ and basal DM contained 20 μ M Ca²⁺. The indicated values are the sums of the basal and added Ca²⁺ concentrations. CSD with zero Ca²⁺ was prepared using ingredients which had been passed over Chelex-100.

Table 2. *Ca²⁺ content of subcellular fractions of microplasmodia grown in medium containing different concentrations of Ca²⁺*

Microplasmodia were grown to late-exponential phase on CSD medium. CaCl₂ was added to CSD as indicated; basal CSD contained 50 μ M-Ca²⁺. Microplasmodia washed in 20 mM-citric acid/NaOH buffer (pH 4.6) were fractionated as described in Methods, with 1 mM-EGTA added to the homogenizing medium. Concentrations of Ca²⁺ are expressed as nmol (mg protein)⁻¹ and are the means and standard errors for three different experiments.

Subcellular fractions	Ca ²⁺ present in growth medium (mM)			
	0.05	0.30	2.55	25.05
Homogenate	34 $\pm$ 14	64 $\pm$ 13	123 $\pm$ 17	416 $\pm$ 18
500 g pellet	31 $\pm$ 10	51 $\pm$ 13	89 $\pm$ 19	282 $\pm$ 27
Mitochondria	20 $\pm$ 6	50 $\pm$ 4	84 $\pm$ 9	215 $\pm$ 19
Vesicles	46 $\pm$ 7	56 $\pm$ 11	95 $\pm$ 22	156 $\pm$ 7
Microsomes	24 $\pm$ 3	45 $\pm$ 18	77 $\pm$ 6	236 $\pm$ 35
Supernatant	47 $\pm$ 11	92 $\pm$ 7	152 $\pm$ 20	626 $\pm$ 51

smaller than those grown in media with added Ca²⁺. The reasons for this are unknown, though Ca²⁺ limitation may have caused microplasmodia to fragment more readily in shaken cultures.

The growth media recommended by Carlile (1971) and Dee *et al.* (1973) contain EDTA. However, the effect of EDTA as a chelating agent at pH 4.6 (the pH of media for culturing *P. polycephalum*) is small. For instance, the apparent association constant of EDTA for Ca²⁺ is decreased from 10^{10.7} to 10^{3.4}, calculated according to Flaschka (1964). The apparent association constant of the chelating agent EGTA for Ca²⁺ is likewise decreased from 10¹¹ to 10^{2.1}. The decreased effectiveness of EDTA and EGTA as Ca²⁺-chelators may account for the observation that they did not affect the growth rate or yield in CSD medium; there was a small effect in defined medium.

Fig. 2. $^{45}\text{Ca}^{2+}$ and $[^3\text{H}]\text{inulin}$ uptake by microplasmodia. At zero time, $10 \mu\text{l}$ $^{45}\text{CaCl}_2$ ($50 \mu\text{Ci ml}^{-1}$) or $40 \mu\text{l}$ $[^3\text{H}]\text{inulin}$ ($50 \mu\text{Ci ml}^{-1}$, 680 Ci mmol^{-1}) was added to 50 ml of exponentially growing culture (MCM, 0.25 mM-Ca^{2+} added, 25°C). Samples were removed at intervals and filtered, washed and assayed for radioactivity as described in Methods. These concentrations of isotopes yielded the same number of counts $(\text{ml medium})^{-1}$ (allowing for differences in efficiency of counting) and permitted direct comparison of rates of uptake as $\text{c.p.m. h}^{-1} (\text{mg protein})^{-1}$. (a) KCN (1 mM) was added 1 min before $^{45}\text{CaCl}_2$ (respiration is inhibited by 97 % in this time): $\circ$, $^{45}\text{Ca}^{2+}$ control; $\bullet$, $^{45}\text{Ca}^{2+}$ plus KCN. (b) KCN (1 mM) was added 1 min before $[^3\text{H}]\text{inulin}$: $\circ$, $^{45}\text{Ca}^{2+}$ control; $\bullet$, $[^3\text{H}]\text{inulin}$ control; $\square$, $[^3\text{H}]\text{inulin}$ plus KCN.

Fig. 3. Effect of external Ca^{2+} concentration on $^{45}\text{Ca}^{2+}$ uptake by microplasmodia. $^{45}\text{Ca}^{2+}$ uptake was measured in MCM cultures (0.25 mM-Ca^{2+} added) as described in Fig. 2 except that carrier CaCl_2 was added to bring the external Ca^{2+} concentration to that indicated.

Variation in intracellular Ca^{2+} with changes in extracellular Ca^{2+}

Microplasmodia grown in medium containing Ca^{2+} at concentrations varying over a 500-fold range varied approximately 10-fold in their Ca^{2+} content (Table 2). Subcellular fractions obtained from homogenates varied in a similar fashion except for the vesicle fraction which showed only a fourfold change. EGTA was included in the homogenizing medium to prevent the Ca^{2+} uptake by subcellular fractions which occurred following homogenization in buffered sucrose medium. However, under these conditions a substantial Ca^{2+} efflux from mitochondria, and possibly other organelles, occurs (Holmes & Stewart, 1979). This could not be controlled by addition of the mitochondrial Ca^{2+} transport inhibitors La^{3+} and ruthenium red.

Table 3. *Inhibitors and stimulators of $^{45}\text{Ca}^{2+}$ uptake by microplasmodia*

For each assay, 3 ml of exponential phase microplasmodial culture growing in MCM was shaken in a 15 ml glass vial for 1 min at 25 °C with the effector before adding $^{45}\text{Ca}^{2+}$ ($0.2 \mu\text{Ci ml}^{-1}$). After 1 h, the $^{45}\text{Ca}^{2+}$ taken up was measured as outlined in Methods. The determinations with errors represent the means and standard errors for three experiments; other experiments are means of duplicate assays.

Effector	$^{45}\text{Ca}^{2+}$ uptake [nmol (mg protein) $^{-1}$]
None	9.0 ± 0.6
KCl (100 mM)	3.1 ± 0.2
MgCl ₂ (5 mM)	8.4 ± 0.3
MnCl ₂ (5 mM)	5.2 ± 0.2
SrCl ₂ (5 mM)	2.2 ± 0.3
LaCl ₃ (0.3 mM)	9.2
KCN (1 mM)	3.5 ± 0.1
Iodoacetate (1 mM)	4.0 ± 0.1
KCN (1 mM) + iodoacetate (1 mM)	4.5 ± 0.5
Br-X537A (5 μM)	9.3
Br-X537A (15 μM)	15.4
Br-X537A (50 μM)	21.0
A23187 (5 μM)	11.6
A23187 (15 μM)	13.8
A23187 (50 μM)	13.1
Cytochalasin B (50 μM)	8.7
Cytochalasin B (500 μM)	6.0

Ca $^{2+}$ uptake by microplasmodia

During the uptake of $^{45}\text{Ca}^{2+}$ from the medium by microplasmodia, there was an initial rapid uptake of about 1 nmol (mg protein) $^{-1}$ before a steady state linear rate of uptake of 6.6 nmol h $^{-1}$ mg $^{-1}$ was established (Fig. 2*a*). Simple saturation kinetics were not seen in plots of the rate of Ca^{2+} uptake against the Ca^{2+} concentration in the medium. A double reciprocal plot (Fig. 3) revealed the biphasic nature of Ca^{2+} uptake, with a saturable and an unsaturable component. The saturable component had an apparent maximum velocity of 67 nmol h $^{-1}$ mg $^{-1}$ and an apparent K_m of 1.9 mM.

The uptake of $^{45}\text{Ca}^{2+}$ was compared with that of [^3H]inulin (mol. wt 3000 to 5000) which is generally thought to be metabolically inert and not transported into cells (Bowers & Olszewski, 1972). [^3H]Inulin was taken up at approximately one-third of the rate of $^{45}\text{Ca}^{2+}$ uptake (Fig. 2*b*). There was also a fast initial uptake before an equilibrium rate was established. Inulin uptake was inhibited approximately 60% by 1 mM-KCN.

Effect of inhibitors on Ca $^{2+}$ uptake

The rate and amount of Ca^{2+} taken up was decreased to 35 to 40% of the control by 1 mM-KCN, suggesting that a proportion of the uptake depends on respiratory energy (Fig. 2*a*; Table 3). Iodoacetate (1 mM) inhibited Ca^{2+} uptake by 55%; this may be a consequence of inhibition of glycolytic energy production, or of inhibition of the transport mechanism itself. KCN and iodoacetate, when added together, inhibited Ca^{2+} uptake less than did KCN alone (Table 3).

The presence of 5 mM-Sr $^{2+}$ in the uptake medium (containing 0.3 mM- Ca^{2+}) inhibited Ca^{2+} uptake, as did 5 mM-Mn $^{2+}$, though to a lesser extent (Table 3). Addition of 5 mM-Mg $^{2+}$ to the medium, which already contained 2.4 mM-Mg $^{2+}$, had no effect (Table 3). K $^{+}$ was inhibitory at 100 mM. La $^{3+}$, a Ca^{2+} antagonist, had no effect on Ca^{2+} uptake up to 0.3 mM. Above this concentration microscopic observations indicated that La $^{3+}$ damaged microplasmodia.

Verapamil, tetracaine and procaine, all local anaesthetics which increase Ca^{2+} permea-

Fig. 4. ⁴⁵Ca²⁺ efflux from microplasmodia. Microplasmodia were grown in MCM (0.25 mM-Ca²⁺ added, 25 °C) for 2 d (5 to 6 generations), with ⁴⁵Ca²⁺ present (2 μ Ci ml⁻¹). Suspended microplasmodia (50 ml) were quickly harvested, washed once with 10 vol. of unlabelled medium, resuspended in 50 ml MCM, and returned to the shaker at 25 °C. Samples (3 ml) were taken at the times indicated and filtered (without washing) as described in Methods. Radioactivity was determined in the filtrate. ○, Control; ●, 1 mM-KCN in resuspension medium.

Fig. 5. Effect of ionophore A23187 on the surface area and protein content of macroplasmodia. Macroplasmodia were established as described in Methods. A23187 (2 μ M) was added to the medium (PRM) at the times indicated (arrows) (zero time was set arbitrarily late in the G2 phase between the first and second mitotic divisions, determined by light microscopy of smears of small pieces taken from the edge of the plasmodia). (a) Protein was measured by scraping the whole of the plasmodium from its filter paper support, homogenizing in 0.05 % (w/v) sodium dodecyl sulphate, 0.025 % (w/v) sodium deoxycholate, 0.15 M-NaCl, 0.01 M-Na₂EDTA, 0.1 M-Tris/HCl (pH 7.4), precipitating a sample with an equal volume of 10 % (w/v) trichloroacetic acid, washing with 10 % (w/v) trichloroacetic acid, redissolving the sample in 1.0 M-NaOH, and assaying protein by Lowry's method. (b) Surface area was determined by taking as diameter, the mean of four cross-measurements of the approximately circular plasmodium. ○, No ionophore; ●, ionophore added in G2 phase of cell cycle; □, ionophore added in M phase; ■, ionophore added in S phase.

bility in cells with excitable surface membranes (Jafferji & Mitchell, 1976), had no effect up to 1 mM on Ca²⁺ uptake by microplasmodia.

Ca²⁺ efflux from microplasmodia

When microplasmodia were preloaded with ⁴⁵Ca²⁺ by growth for 2 d in medium containing ⁴⁵Ca²⁺ (2 μ Ci ml⁻¹), a biphasic pattern of ⁴⁵Ca²⁺ efflux was observed on transfer to unlabelled medium (Fig. 4). An initial rapid rate of efflux occurred for up to 10 min during which 10 % of the microplasmodial ⁴⁵Ca²⁺ was released into the medium. This was followed by a lower, linear rate of efflux accounting for about 8 % of the microplasmodial ⁴⁵Ca²⁺ released into the medium per hour. KCN increased the efflux.

Fig. 6. Effect of ionophore A23187 on DNA and protein synthesis in microplasmodia. Samples (50 ml) of microplasmodial suspension, growing exponentially in PRM (5 mM- Ca^{2+}) were harvested, resuspended in 10 ml PRM (minus tryptone and yeast extract) containing [^{14}C]lysine (1 $\mu\text{Ci ml}^{-1}$) and [^3H]thymidine (2 $\mu\text{Ci ml}^{-1}$), and shaken at 25 $^{\circ}\text{C}$. At the times indicated, duplicate 0.5 ml samples were withdrawn and added to an equal volume of cold 10% (w/v) trichloroacetic acid. After 30 min on ice, samples were filtered through glass-fibre discs and washed with 3×5 ml cold 10% trichloroacetic acid, 0.1% (w/v) thymidine, 0.1% (w/v) lysine, then twice with 5 ml ethanol. After drying, ^3H and ^{14}C radioactivity on the filters was determined. Protein was determined on separate samples as described in Fig. 5. (a) [^{14}C]Lysine incorporated; (b) [^3H]thymidine incorporated. $\circ$, Control; $\square$, 20 μM -A23187 in medium; $\bullet$, 40 μM -A23187 in medium.

Effect of ionophores on growth and metabolism

The ionophores A23187 and Br-X537A change the permeability properties of membranes towards divalent cations allowing the equilibration of Ca^{2+} across membranes (Truter, 1976). Both ionophores stimulated Ca^{2+} uptake, Br-X537A being more effective (Table 3). The effect of A23187 on mitotically synchronous macroplasmodia was examined in further detail since Duffus & Patterson (1974) have reported that in the yeast *Schizosaccharomyces pombe* the cell cycle was blocked in or near mitosis following the addition of the ionophore.

When *P. polycephalum* macroplasmodia were exposed to A23187 (20 or 40 μM) gross morphological changes were observed. The protoplasm contracted within 20 to 30 min to produce a network of thin veins, and the size of the plasmodium did not increase over the next 24 h. When this effect was quantified by measuring the change in surface area and protein content (Fig. 5), the qualitative observations on plasmodial growth were confirmed. The same effect was observed whether the ionophore was added in the S, G2 or mitotic phases of the cell cycle.

Mitoses were not observed when the ionophore was added in the G2 or S phases. It was difficult to determine whether it occurred when the ionophore was added during mitosis as nuclei became structurally aberrant. Nuclei at all stages of the cell cycle became enlarged and developed multiple nucleoli on prolonged incubation with the ionophore. When a microplasmodial suspension was layered on to CSD agar containing 20 μM -A23187, a macroplasmodium did not develop. Instead plasmodia contracted to form a small ball, suggesting that strong contractions of the protoplasm followed microplasmodial fusion.

To determine whether A23187 affected macromolecular synthesis related to the cell cycle and mitosis, microplasmodial uptake of [^3H]thymidine and [^{14}C]lysine into DNA and protein, respectively, was examined. No inhibition was observed with 20 μM -A23187 which produced the morphological and structural effects described earlier. However, the incorporation of both isotopes was inhibited by 40 μM -A23187 (Fig. 6).

A23187 at 20 μM stimulated microplasmodial respiration for 30 min, which then returned to a control rate, whereas at 40 μM it almost completely inhibited respiration (results not shown). The higher ionophore concentration could thus be exerting its metabolic effects through an inhibition of mitochondrial energy production.

DISCUSSION

In the only other study of the relationship of Ca²⁺ to growth in *Physarum polycephalum*, Daniel & Rusch (1961) demonstrated that little growth occurred without added Ca²⁺. The amount of Ca²⁺ in their medium would, however, have been at least 300 μ M based on the Ca²⁺ content of the medium components (tryptone, yeast extract and glucose). Experiments reported here demonstrate that the Ca²⁺ concentration of the medium had to be less than 50 μ M before an effect on growth could be observed. It was necessary to remove Ca²⁺ from the major medium components with a Ca²⁺-chelating resin before a requirement for Ca²⁺ could be demonstrated.

Microplasmodia grown with added Ca²⁺ at 0.25 to 2.5 mM contained 50 to 120 nmol Ca²⁺ (mg protein)⁻¹ (Table 2). Nagai *et al.* (1975), by contrast, reported 20 to 45 nmol Ca²⁺ (mg fresh wt)⁻¹ for plasmodia grown by the method of Camp (1936). Assuming that wet ('fresh') plasmodia contain approximately 5% (w/w) protein, this value becomes 400 to 500 nmol (mg protein)⁻¹, i.e. about sevenfold higher than our values. We have no explanation for this difference.

Plasmodia show a partial Ca²⁺ homeostasis since their Ca²⁺ content changed only 10-fold when the medium Ca²⁺ concentration was varied 500-fold. The high Ca²⁺ content of the mitochondrial and microsomal fractions at high external Ca²⁺ concentration indicated a capacity to concentrate Ca²⁺. Free Ca²⁺ concentrations in the cytoplasm of *P. polycephalum* are in the micromolar range (Hatano, 1970; Ridgway & Durham, 1976). The high concentration of Ca²⁺ (about 1 mM) in the postmicrosomal supernatant argues either for substantial Ca²⁺ release from particulate components such as mitochondria, the endoplasmic reticulum or Ca²⁺-containing vesicles, or the presence of large quantities of Ca²⁺-binding compounds in the cytosol of *P. polycephalum* plasmodia.

Although the inclusion of EGTA in the homogenizing medium may have released Ca²⁺ from some compartments, high Ca²⁺ concentrations were also found in the soluble fraction when EGTA was omitted. The centrifugation of microplasmodia directly, or variation of osmolarity, ionic strength or pH of the homogenizing medium, or the inclusion of La³⁺ or ruthenium red in an attempt to limit Ca²⁺ redistribution, were likewise without significant effect on Ca²⁺ recovered in the post-microsomal supernatant. Thus, either damage is sustained by Ca-sequestering organelles during homogenization or efflux occurs as a consequence of change in the environmental conditions of the organelles. Until Ca²⁺ redistribution is better understood, measurement of Ca²⁺ in subcellular compartments by fractionation and direct assay is not a particularly fruitful approach.

Substantial Ca²⁺ uptake and release were detected in microplasmodia demonstrating that Ca²⁺ fluxes occur in both directions across the plasma membrane. Such transitory changes may be important during cytoplasmic streaming and mitosis (Holmes & Stewart, 1977). Furthermore, extracellular Ca²⁺ and oxygen uptake were closely coordinated during mitosis, indicating that mitochondria may be involved in sequestration or energy production during the cyclical uptake of extracellular Ca²⁺. *Physarum polycephalum* mitochondria have been shown to take up Ca²⁺ actively (Holmes & Stewart, 1979).

The rate of Ca²⁺ uptake by microplasmodia was partly saturable with an apparent K_m of 1.9 mM. However, the method used to determine the rate of Ca²⁺ uptake may underestimate the initial velocity of the process, and thus the measure of saturation given by the apparent K_m may be an overestimate.

The divalent ions Sr²⁺ and Mn²⁺ inhibited Ca²⁺ uptake, consistent with similar competitive inhibition in other systems. Both saturation kinetics and inhibition by other divalent ions are compatible with Ca²⁺ uptake occurring through a fixed number of specific sites on the cell surface. However, carrier-mediated transport, membrane pores, or absorptive pinocytosis, could all provide such saturable sites and further experiments are required to differentiate between possible mechanisms.

Physarum polycephalum is an actively pinocytotic organism (Guttes & Guttes, 1961; Daniel & Jarlfors, 1972). Assuming that uptake of [^3H]inulin occurs by pinocytosis, this process could account for approximately 30% of the Ca^{2+} taken up. As significant amounts of inulin are not known to bind to cell surfaces, the uptake of inulin may represent bulk-phase pinocytosis whereby droplets of medium are ingested. Such a form of pinocytotic uptake would be unsaturable and consistent with the unsaturable phase of Ca^{2+} uptake detected.

The ionophores A23187 and Br-X537A have been useful tools in elucidating the role of Ca^{2+} in cellular metabolism (Truter, 1976). A23187 did not block plasmodia in the mitotic stage of the cell cycle as has been reported for yeast (Duffus & Patterson, 1974). Nevertheless, the inhibition of plasmodial diameter increase, the contractile responses induced in synchronized plasmodia and during plasmodial initiation, and the morphological changes in nuclei produced by low concentrations of A23187 indicate that the ionophore has significant effects on the organism. The effect of Br-X537A was more potent than A23187 in stimulating Ca^{2+} influx but its effect on plasmodial morphology was not as great as that of A23187. This may be a consequence of the low pH of the growth medium which would affect the ionophoretic properties of A23187 (Pfeiffer & Lardy, 1976).

This work was supported in part by the Australian Research Grants Committee.

REFERENCES

- BALK, S. D., WHITFIELD, J. F., YOUNDALE, Y. & BRAUN, A. C. (1973). Roles of calcium, serum, plasma and folic acid in the control of proliferation of normal and Rous sarcoma virus-infected chicken fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* **70**, 675–679.
- BERRIDGE, M. J. (1975). The interaction of cyclic nucleotides and calcium in the control of cellular activity. *Advances in Cyclic Nucleotide Research* **6**, 1–98.
- BORLE, A. B. (1973). Calcium metabolism at the cellular level. *Federation Proceedings* **32**, 1944–1950.
- BOWERS, B. & OLSZEWSKI, T. E. (1972). Pinocytosis in *Acanthamoeba castellanii*. Kinetics and morphology. *Journal of Cell Biology* **53**, 681–694.
- CAMP, W. G. (1936). A method of cultivating myxomycete plasmodia. *Bulletin of the Torrey Botanical Club* **63**, 206–210.
- CARLILE, M. J. (1971). Myxomycetes and other slime moulds. *Methods in Microbiology* **4**, 237–265.
- DANIEL, J. W. & RUSCH, H. P. (1961). The pure culture of *Physarum polycephalum* on a partially defined soluble medium. *Journal of General Microbiology* **25**, 47–59.
- DANIEL, J. W. & BALDWIN, H. H. (1964). Methods of culture for plasmodial myxomycetes. *Methods in Cell Physiology* **1**, 9–41.
- DANIEL, J. W. & JARLFORS, U. (1972). Plasmodial ultrastructure of the myxomycete *Physarum polycephalum*. *Tissue and Cell* **4**, 15–36.
- DEE, J., WHEALS, A. E. & HOLT, C. E. (1973). Inheritance of plasmodial valine requirement in *Physarum polycephalum*. *Genetical Research* **21**, 87–101.
- DUFFUS, J. H. & PATTERSON, L. J. (1974). Control of cell division in yeast using the ionophore A23187 with calcium and magnesium. *Nature, London* **251**, 626–627.
- FLASCHKA, H. A. (1964). *EDTA Titrations*. Oxford: Pergamon Press.
- GUTTES, E. & GUTTES, S. (1964). Mitotic synchrony in the plasmodia of *Physarum polycephalum* and mitotic synchronization by coalescence of microplasmodia. *Methods in Cell Physiology* **1**, 43–54.
- HATANO, S. (1970). Specific effect of Ca^{2+} on movement of plasmodial fragments obtained by caffeine treatment. *Experimental Cell Research* **61**, 199–203.
- HOLMES, R. P. & STEWART, P. R. (1977). Calcium uptake during mitosis in the myxomycete *Physarum polycephalum*. *Nature, London* **269**, 592–594.
- HOLMES, R. P. & STEWART, P. R. (1979). The isolation of coupled mitochondria from *Physarum polycephalum* and their response to Ca^{2+} . *Biochimica et biophysica acta* **545**, 94–105.
- JAFFERJI, S. S. & MITCHELL, R. H. (1976). Effects of calcium-antagonistic drugs on the stimulation by carbamoylcholine and histamine of phosphatidylinositol turnover in longitudinal smooth muscle of guinea-pig ileum. *Biochemical Journal* **160**, 163–169.
- KATO, T. & TONOMURA, Y. (1977). Uptake of calcium ions into microsomes isolated from *Physarum polycephalum*. *Journal of Biochemistry* **81**, 207–213.
- NAGAI, R., ISHIMA, Y., KIKITA, F. & TAKENAKA, T. (1975). Calcium and magnesium contents of ectoplasm and endoplasm of *Physarum polycephalum* plasmodium. *Protoplasma* **86**, 169–174.
- PFEIFFER, D. R. & LARDY, H. A. (1976). Ionophore A23187: the effect of H^+ concentration on complex formation with divalent and monovalent

- cations and the demonstration of K⁺ transport in mitochondria mediated by A23187. *Biochemistry* **15**, 935–943.
- RASMUSSEN, H. (1975). Ions as “second messengers”. In *Cell Membranes in Biochemistry, Cell Biology and Pathology*, pp. 203–212. Edited by G. Weismann & R. Clairborne. New York: H. P. Publishing.
- RIDGWAY, E. B. & DURHAM, A. C. H. (1976). Oscillations of calcium ion concentrations in *Physarum polycephalum*. *Journal of Cell Biology* **69**, 223–226.
- TRUTER, M. R. (1976). Chemistry of the calcium ionophores. *Symposia of the Society for Experimental Biology* **30**, 19–40.