Calcium transport in the cellular slime mould *Dictyostelium discoideum*

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Transport of Ca^{2+} into amoebae of *Dictyostelium discoideum* was studied using ^{45}Ca and a lanthanum stopping technique. Ca^{2+} uptake was found to be rapid and showed saturation kinetics. No difference was found in Ca^{2+} uptake between vegetative and aggregation competent cells, the V_{max} for unstimulated amoebae being approx. 10 nmol/10^7 cells per min. Ca^{2+} uptake had the characteristics of passive facilitated diffusion using a saturatable carrier and NaN_3 and ouabain were not inhibitory. The chemoattractants cAMP and folate, previously reported to stimulate the uptake of Ca^{2+} into amoebae, did not stimulate the rate of Ca^{2+} uptake by this carrier but increased the extent of Ca^{2+} taken up over the period 10–30 s after chemotactic stimulation. The significance of these findings for the function of Ca^{2+} in chemotactic signalling is discussed.

Calcium Dictyostelium *Folate* *Cyclic AMP* *Chemotaxis*

1. INTRODUCTION

Amoebae of the cellular slime mould *Dictyostelium discoideum* show a chemotactic response to folate during the vegetative phase and to cAMP during the aggregation phase. Studies of this chemotactic process have identified several cellular events that occur within a few seconds of binding of the chemoattractant to chemoattractant-specific cell surface receptors [1]. One of these responses is the rapid influx of Ca^{2+} from the surrounding medium. Studies by Wick et al. [2] and by Bumann et al. [3] indicated that in both the vegetative and aggregation-competent stages an increased influx of Ca^{2+} into amoebae is observed when these cells are stimulated with folate or cAMP (respectively). These studies, however, did not determine any properties of the receptor involved or the kinetic parameters of normal and stimulated uptake.

It is important, however, to determine the time course and kinetics of Ca^{2+} uptake to understand the role of this process in the rapid events of chemotactic signalling. In the present investigation, we sought to characterise the Ca^{2+} transport process to see whether such transport is an active process or uses a passive facilitated carrier, and to determine the timing of the changes stimulated by the chemoattractants folate and cAMP.

2. MATERIALS AND METHODS

Arsenazo III was from Sigma (London), Poole, England. $^{45}Ca^{2+}$ (10 mCi/mg Ca; 0.37 GBq/mg Ca) was obtained from Amersham International, Bucks. Nutrient 'SM' agar of Sussman [4] was prepared according to Mosses et al. [5]. The wild-type strain NC4 of *D. discoideum* was grown in association with *Klebsiella aerogenes* (strain OXF1) on SM agar in the dark at 22°C.

Calcium-free salt solution (CFSS) contained 0.6 g NaCl, 0.75 g KCl, distilled, deionized water to 1 l. Phosphate buffer (PB) contained: 17 mM Na/K phosphate, pH 6.1.

2.1. *Preparation of amoebae for Ca^{2+} transport*

Amoebae were harvested from SM agar plates after 24 h growth as the amoebae cleared the

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bacterial lawn and were washed free from bacteria by centrifugation in PB (3 spins at 190 × *g* for 2 min). For Ca^{2+} transport using vegetative cells, amoebae were washed in CFSS and suspended in CFSS at 10^8 ml^{-1}. For Ca^{2+} transport in aggregation competent cells, amoebae were preincubated as a suspension at 10^7 ml^{-1} in PB for 5 h in a rotary incubator (170 rpm) before being centrifuged, washed in CFSS and resuspended in CFSS at 10^8 ml^{-1}.

2.2. *Transport of Ca^{2+} into amoebae*

Transport was initiated by the rapid addition of $CaCl_2$ containing radioactive $^{45}Ca^{2+}$ tracer (10 μCi/ml) to 100 μl of cell suspension shaking in 1.5-ml Eppendorf tubes at 22°C on an IKA-Vibrax rotary platform shaker at 1400 rpm to give Ca^{2+} concentrations ranging from 0.46 to 2.27 mM. Mixing occurred in less than 0.1 s as judged from the mixing of dyes in control experiments. Transport was stopped at the appropriate times by the rapid addition of 100 μl of 320 μM $LaCl_3$ (giving a final concentration of 100 μM). The tubes were centrifuged in a Beckman microfuge at 8000 × *g* and after removal of the supernatant the cell pellets were washed with 1 ml of cold (CFSS) to eliminate trapped Ca^{2+} and recentrifuged at 8000 × *g*. (No further washing was required as the number of CPMs obtained did not decrease when the cell pellets were washed more than once.) Zero time points were taken by addition of $LaCl_3$ to a final concentration of 100 μM prior to addition of labelled Ca^{2+} solution.

The effects of folate and cAMP on Ca^{2+} transport were obtained by adding the respective chemoattractants to cells at the same time as labelled Ca^{2+} solution. Initial rates were estimated from the slope of the progress curves from time zero to 5 s.

2.3. *Determination of Ca^{2+} concentration*

Calcium concentrations were determined using the absorption difference of 100 μM arsenazo III at 660 and 685 nm [6]. Samples (100 μl) of the extracellular medium and cell sediment were deproteinized with 100 μl of 3.5% (v/v) $HClO_4$ and then neutralised with 50 μl of 50% saturated $KHCO_3$ to give pH 7–8. A calibration curve was constructed and reagent blanks subtracted from sample values.

3. RESULTS AND DISCUSSION

3.1. *Conditions for use of lanthanum in stopping Ca^{2+} transport*

$LaCl_3$ has been used to stop Ca^{2+} transport in several tissues such as pancreatic islets [7], rabbit aorta [8] and mouse exocrine pancreas [9]. Using *D. discoideum*, Malchow has found that 100 μM $LaCl_3$ is sufficient to inhibit Ca^{2+} uptake (unpublished data cited by Bumann [3]). To confirm that this concentration was optimal, the effects of $LaCl_3$ concentrations ranging from 25 to 200 μM were investigated. It was found that below 75 μM, $LaCl_3$ did not inhibit Ca^{2+} transport (as measured by ^{45}Ca uptake and release) whereas above 150 μM it caused cell lysis. At a concentration of 100 μM, $LaCl_3$ effectively inhibited Ca^{2+} transport but caused no cell lysis, and this concentration was used subsequently in all further experiments.

3.2. *Intracellular and extracellular Ca^{2+} concentrations*

Prior to studying Ca^{2+} transport, cellular and extracellular Ca^{2+} concentrations were determined using arsenazo III. Amoebae were harvested from SM growth plates, separated from the bacterial food source and 2×10^8 cells·ml^{-1} allowed to equilibrate in CFSS for 30 min. Ca^{2+} was determined in the complete suspension, the packed cells after centrifugation and the supernatant. (The volume of packed cells was approx. 15% of the total volume of the cell suspension.) The results (± SE of 4 experiments) showed a mean cellular concentration of 0.83 ± 0.19 mM equilibrated in an extracellular concentration of 0.53 ± 0.12 mM. The finding of large cellular Ca^{2+} stores in these cells, which confirms the results of Wick et al. [2], is presumably due to Ca^{2+} sequestered in membrane-bound subcellular compartments or organelles. Determination of the extracellular concentration at equilibrium was important for ensuring that the effects of chemoattractants (see below) were observed using physiologically normal extracellular concentrations.

3.3. *Ca^{2+} uptake occurs by facilitated diffusion*

The rate of Ca^{2+} uptake into amoebae was determined using $^{45}Ca^{2+}$ with 100 μM $LaCl_3$ to stop uptake after periods of 0–60 s. The initial rate of Ca^{2+} uptake (over the first 5 s) was rapid and

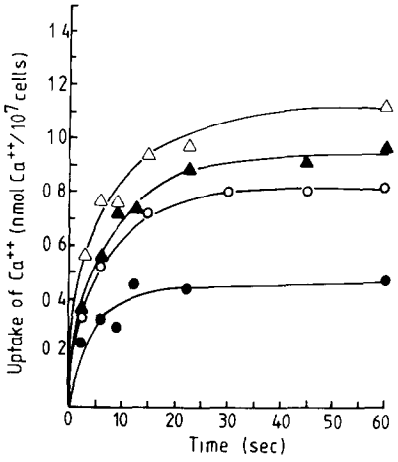

Fig.1. Uptake of Ca^{2+} into amoebae of *D. discoideum*. Measurements were carried out at 22°C using aggregation competent amoebae with extracellular Ca^{2+} concentrations of 0.46 mM (●), 1.14 mM (○), 1.71 mM (▲) and 2.27 mM (△). Data points represent the means of 5 experiments.

began to equilibrate after about 25 s (fig.1). Counts taken up at equilibrium represented a 100% increase of Ca^{2+} bound or trapped at time zero. Uptake increased with increasing Ca^{2+} concentrations in the extracellular medium. When the data (from 4 experiments) were plotted as initial rates of uptake against Ca^{2+} concentration the curve showed Michaelis-Menten saturation, indicating the involvement of a saturatable transport system. Double-reciprocal plots (fig.2) showed a

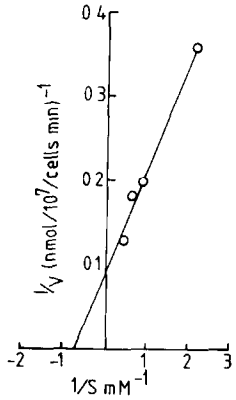

Fig.2. Double-reciprocal plots of initial rates of Ca^{2+} uptake into aggregation-competent amoebae. Lines were fitted by regressional analysis. Each point is the mean of data from 5 experiments.

V_{max} of approx. 10 nmol·10^7 cells^{-1}·min^{-1}. To obtain statistics, the V_{max} was also calculated by plotting the results of 5 individual experiments separately and after line fitting by regressional analysis, determining the V_{max} values ± SE. This gave a V_{max} value of 8.5 ± 1.4 nmol·10^7 cells^{-1}·min^{-1}. An apparent K_m value of 1.4 mM was also estimated from these plots, although the significance of such an apparent K_m value for a complex system such as ion transport is open to question. No difference in Ca^{2+} uptake was observed between vegetative and aggregation-competent cells.

To determine whether such Ca^{2+} uptake was active or passive the amoebae were pre-incubated with the respiratory inhibitor NaN_3. (The amoebae use a conventional cyanide-sensitive electron transport chain during late growth phase and the aggregation stage [10].) NaN_3 at 5 mM incubated for 10 min with amoebae had no effect on Ca^{2+} uptake, indicating that Ca^{2+} transport does not require ATP hydrolysis. The system may therefore be said to be a facilitated process rather than an active one.

Cells treated for 5 min with 1 mM ouabain prior to Ca^{2+} uptake studies also showed no inhibition of transport indicating that transport, does not require a functioning Na^+/K^+ ATPase.

3.4. *Chemotactic agents stimulate the extent but not the rate of Ca^{2+} uptake*

When vegetative and aggregation competent cells were treated with folate and cAMP, respectively, they showed markedly increased uptake of Ca^{2+} measured at the equilibrium plateau values, 30–60 s after stimulation (table 1). This increase in the cellular Ca^{2+} concentration in cAMP-stimulated amoebae was calculated to be 77 μM in 30 s (table 1) which agrees closely with the results of Bumann et al. [3] who calculated a figure of 60 μM over 30 s from measurements of loss of Ca^{2+} from the medium using a Ca^{2+} electrode.

This increased uptake into chemotactically stimulated cells was not due, however, to any significant increase in the rate of Ca^{2+} uptake, but was accounted for by an increase in the extent of uptake over the period 10–30 s after stimulation (fig.3). Using extracellular Ca^{2+} concentrations ranging from 0.46 to 1.17 mM, no significant differences were observed in the initial rates of Ca^{2+}

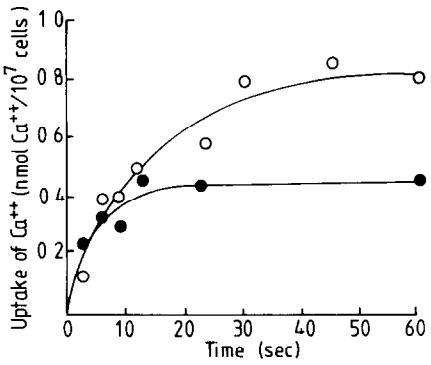

Fig.3. Stimulation of Ca^{2+} uptake by cAMP. Measurements of Ca^{2+} uptake from an extracellular concentration of 0.46 mM were made at 22°C using aggregation-competent amoebae. (●) Controls, (○) amoebae stimulated with 50 nM cAMP (final concentration) at time zero. Each point is the mean of data from 5 experiments (controls) or 3 experiments (cAMP stimulated).

uptake between cells stimulated with cAMP or folate and unstimulated controls.

The significance of this finding is that it eliminates extracellular Ca^{2+} uptake as a candidate for the primary response after chemotactic stimulation. The binding of cAMP or folate to the cell surface receptors triggers the rapid polymerisation of actin and its association with the cytoskeleton [11,12] a response thought to be connected with subsequent chemotactic movement. As this response is seen to peak at 3–5 s after binding of chemoattractants, any intermediate messenger between the receptors and the actin would have to be stimulated over a similar timescale. The data presented here show that the Ca^{2+} uptake response shows no significant change over that period, but rather a slower effect over 10–30 s which is well after the primary actin response has occurred and a pseudopodium has been produced in the chemotactically stimulated amoeba. This conclusion is consistent with the finding that brief incubation with high extracellular EDTA [13] or EGTA [14] concentrations does not inhibit chemotaxis and tends to support the results [14,15] suggesting that liberation of Ca^{2+} from the large intracellular stores is the most likely source of intracellular Ca^{2+} signalling.

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Table 1

Stimulation by cAMP and folate of the equilibrium level for Ca^{2+} uptake

Additions	Extracellular Ca^{2+} concentration (mM)	% increase in equilibrium Ca^{2+} level over control	Calculated increase in cellular Ca^{2+} concentration over control (μM)
Control	0.46	–	–
cAMP	0.46	89 ± 12	77
Folate	0.46	45 ± 5	39

Uptake of 0.46 mM $^{45}Ca^{2+}$ was measured in washed vegetative phase amoebae stimulated with 50 μM folate and in aggregation competent starving amoebae with 50 nM cAMP (final concentrations). Uptake curves in each case showed that equilibrium was reached at about 30 s and remained constant to 60 s. Data are the mean of 4 experiments (± SE). The calculated increase in cellular Ca^{2+} concentration uses a figure for the cell volume of 5.2×10^{-10} cm^3 (r = 5 μm)

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