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Isolation and characterization of a plasma membrane calcium pump from *Dictyostelium discoideum*

Jacqueline L. MILNE and M. Barrie COUKELL
Department of Biology, York University, 4700 Keele Street, North York, Ontario M3J 1P3, Canada

During the aggregation and differentiation of amoebae of *Dictyostelium discoideum*, changes in free cytosolic Ca^{2+} appear to regulate a number of physiological processes. To understand the mechanisms regulating free intracellular Ca^{2+} in this organism, we have isolated and characterized an ATP/Mg^{2+}-dependent, high-affinity Ca^{2+} pump. When homogenates of 2 h starved cells were fractionated on Percoll/KCl gradients, one peak of high-affinity Ca^{2+}-pumping activity was detected. This activity was resolved from enzyme markers of the mitochondrion and the rough endoplasmic reticulum but it cosedimented with the plasma membrane marker, alkaline phosphatase. Further studies suggested that the pump was associated with 'inside-out' plasma membrane vesicles. Like plasma membrane Ca^{2+}-transport ATPases from other systems, this isolated Ca^{2+} pump: (1) was Mg^{2+}-dependent, (2) displayed a high specificity for ATP as an energy source, (3) exhibited a high affinity for free Ca^{2+} with a K_m of 0.3 μM, and (4) was very sensitive to inhibition by vanadate (IC_{50} 2 μM) but was unaffected by mitochondrial inhibitors, ouabain and Ca^{2+}-channel blockers. Unlike plasma membrane Ca^{2+} pumps from most other systems, this enzyme appeared not to be regulated by calmodulin. During development, non-mitochondrial, vanadate-sensitive, high-affinity Ca^{2+}-pumping activity in crude lysates remained relatively constant for at least 15 h. These observations suggest that this plasma membrane Ca^{2+} pump probably functions in *Dictyostelium* to maintain Ca^{2+} homeostasis by extruding free cytosolic Ca^{2+} from the cells.

INTRODUCTION

Upon starvation, amoebae of the cellular slime mould *Dictyostelium discoideum* undergo a complex array of developmental events including aggregation, differentiation, and morphogenesis, and eventually form a multicellular fruiting body (for a review, see Loomis, 1982). Growth phase amoebae respond chemotactically to folate, while aggregation-competent cells are stimulated by cyclic AMP (Varnum & Soll, 1981). Association of either chemoattractant with its respective surface receptor elicits a number of intracellular events including actin polymerization (McRobbie & Newell, 1984), dephosphorylation of myosin heavy chain (Malchow *et al.*, 1981), a rapid, transient increase in cyclic GMP (Mato *et al.*, 1977), and an influx of extracellular Ca^{2+} (Wick *et al.*, 1978; Bumann *et al.*, 1984).

In higher eukaryotes, the mechanisms of Ca^{2+} signal transduction and the function of Ca^{2+} as an intracellular regulator are reasonably well understood. While much less is known about Ca^{2+} metabolism in *Dictyostelium*, a number of observations suggest that this ion might be involved in regulating a variety of developmental processes. First, treatment of early developing amoebae with millimolar concentrations of EGTA prevents cell aggregation (Europe-Finner *et al.*, 1984) and also inhibits induction of the cyclic AMP phosphodiesterase by cyclic AMP (Yamasaki & Hayashi, 1982). Second, 100 μM-$LaCl_3$, which effectively inhibits Ca^{2+} uptake (Europe-Finner & Newell, 1985), blocks the differentiation of starved amoebae (Bumann *et al.*, 1984). Third, chemotaxis and cell differentiation are impaired by a variety of Ca^{2+} and calmodulin antagonists (Jamieson & Frazier, 1981; Schaap *et al.*, 1986; M. B. Coukell & A. M. Cameron, unpublished work). Fourth, cyclic AMP receptor-regulated processes associated with chemotaxis such as actin polymerization (Europe-Finner & Newell, 1986*a*), the transient synthesis of cyclic GMP (Small *et al.*, 1986) and calmodulin-dependent inactivation of myosin heavy chain kinase (Malchow *et al.*, 1981; Maruta *et al.*, 1983) are affected *in vitro* (or in saponin-permeabilized cells) by micromolar concentrations of exogenous Ca^{2+}. Finally, it has been reported that high concentrations of extracellular Ca^{2+} induce stalk cell differentiation and inhibit spore formation (Maeda, 1970), and that prestalk regions of migrating slugs preferentially accumulate Ca^{2+} (Maeda & Maeda, 1973). Taken together, these observations suggest that the levels and distribution of cell Ca^{2+} change during development and that there must be cellular mechanisms involved in regulating these changes and maintaining Ca^{2+} homeostasis.

Recent evidence indicates that the concentration of free cytosolic Ca^{2+} in *Dictyostelium* might be regulated, at least in part, by Ca^{2+} pumps. For example, Bumann *et al.* (1984) observed that the addition of folate or cyclic AMP to suspensions of amoebae caused a transient influx of extracellular Ca^{2+} followed shortly by Ca^{2+} extrusion. Although the nature of the Ca^{2+} extrusion system was not investigated further, the authors speculated that it might involve a Ca^{2+} pump. Furthermore, since the cytosolic Ca^{2+} concentration of resting aggregation-competent amoebae was estimated in this study to be about 300 nM, this system would appear to have a high affinity for Ca^{2+}. In addition to this putative plasma membrane pump, Europe-Finner & Newell (1986*b*)

Abbreviations used: IP_3, potassium D-*myo*-inositol trisphosphate; pNPP, sodium *p*-nitrophenylphosphate; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TFP, trifluoperazine hydrochloride; HAMK buffer, 20 mM-Hepes/NaOH/100 mM-KCl/5 mM-$MgCl_2$/1.9 mM-Na_2ATP, pH 7.2.

reported the uptake of $^{45}Ca^{2+}$ into intracellular, non-mitochondrial stores of saponin-permeabilized amoebae by a high-affinity transport system. During an earlier study, Mato & Marín-Cao (1979) detected an ATP-dependent Ca^{2+} pump in crude cell homogenates of *D. discoideum*. In the present investigation, we have extended this work by isolating and characterizing a high-affinity Ca^{2+} pump which appears to be associated with the plasma membrane of this organism.

EXPERIMENTAL

Materials

$^{45}CaCl_2$ (9.71–12.4 mCi/mg of Ca^{2+}; 1 μCi = 37 GBq) was obtained from ICN Biomedical Inc. Hepes, Tris, EGTA, sodium salts of ATP, GTP, CTP, ITP and UTP, bovine brain calmodulin, bovine serum albumin (fraction V), phosphocreatine, creatine phosphokinase, apyrase, calmidazolium, IP_3, 2,6-dichlorophenolindophenol, pNPP, CCCP, Ruthenium Red, ouabain, verapamil, diltiazem and Antifoam B were purchased from Sigma. Sodium metavanadate was from BDH and Percoll was obtained from Pharmacia. Protease peptone was purchased from Difco and yeast extract from Scott Laboratories. All other chemicals were analytical grade and were obtained from local suppliers. TFP was a gift from Smith, Kline & French (Canada) Ltd.

Organism and culture conditions

The axenic mutant AX2 (Watts & Ashworth, 1970) of *D. discoideum* was used throughout this study. Amoebae inoculated into HL-5 medium (Cocucci & Sussman, 1970) supplemented with 200 μg of dihydrostreptomycin/ml and 0.01% Antifoam B were shaken at 250 rev./min on a New Brunswick gyratory shaker at room temperature (22 °C) and grown until the cell density reached (1–1.5) $\times 10^7$ cells/ml. Cells were harvested by centrifugation at 700 *g* for 2 min. Development was initiated by washing the amoebae twice in cold Sorenson's phosphate buffer (17 mM-Na_2HPO_4/KH_2PO_4, pH 6.1), resuspending the cells in the same buffer to a concentration of 2×10^7 cells/ml and shaking the suspension as described above.

Fractionation of crude cell membranes

In preliminary experiments, Ca^{2+}-pumping activity was characterized in crude cell extracts using conditions similar to those of Mato & Marín-Cao (1979). In subsequent experiments, Ca^{2+} transport was studied with membrane preparations fractionated on Percoll/KCl gradients. Initially, the method of O'Rourke *et al.* (1985) was employed to disrupt the cells and fractionate the membranes. Briefly, cells developed for 2 h were washed twice in 10 mM-Tris/HCl/6% (w/v) sucrose, pH 7.5, and resuspended to a concentration of about 5×10^8/ml in 3 ml of HAMK buffer. The cells were then sonicated (Branson Sonifier model W185, microtip, setting 4) three times for 5 s with a 20 s interval on ice between pulses. After centrifugation at 4 °C to remove intact cells, 3 ml of the supernatant was pipetted into a 15 ml glass Corex tube containing 8 ml of 30% Percoll in HAMK buffer. The mixture was centrifuged at 40000 *g* for 35 min in a Sorvall Model RC-5 centrifuge (SS-34 rotor) at 4 °C. Eleven 1 ml fractions were withdrawn from the tube (beginning at the top of the gradient), placed on ice and assayed for Ca^{2+} uptake and various membrane markers. While ATP-dependent Ca^{2+}-transporting activity was found consistently in certain gradient fractions (see under 'Results'), the stability of the transport system prepared in this way varied from experiment to experiment. Subsequent experiments revealed that this activity was considerably more stable when the cells were disrupted by a modification of the filter-breakage method of Das & Henderson (1983). To prepare lysates in this way, 2 h starved cells were washed twice in 20 mM-Hepes/NaOH, pH 7.2, resuspended to about 5×10^8 cells/ml in 3 ml of the same buffer and disrupted by forced passage through two Nuclepore filters (pore size 5 μm) (Nuclepore Corp., Pleasanton, CA, U.S.A.) in a Sartorius membrane filter holder attached to a 10 ml syringe. Unbroken cells and nuclei were removed by centrifugation (4 °C) at 3000 *g* for 2 min in a SS-34 rotor and 3 ml of the supernatant was centrifuged on Percoll/KCl gradients after adjusting the final concentrations of KCl and $MgCl_2$ (ATP was omitted) to those used in earlier gradients. Under these breakage conditions, ATP-dependent Ca^{2+}-transport in crude homogenates and gradient fractions was completely stable for at least 60 min.

Calcium transport assay

Ca^{2+} transport into membrane vesicles was measured using a modification of the filtration assay described by Mato & Marín-Cao (1979). Unless otherwise indicated, the uptake medium contained 20 mM-Hepes/NaOH, pH 7.2, 2 mM-Na_2ATP, 5 mM-$MgCl_2$, 6% sucrose, 0.45 mM-EGTA, 0.33 mM-$CaCl_2$, 100 μM-NaN_3, 100 μM-dinitrophenol, 12 μM-Ruthenium Red, 10 μM-CCCP and approx. 1–2 μCi of $^{45}Ca^{2+}$/ml. Under these conditions, the free Ca^{2+} concentration was 0.55 μM. For kinetic studies, free Ca^{2+} concentrations were set between 0.05 and 2 μM by using Ca^{2+}–EGTA buffers. These buffers contained 0.5 mM-EGTA and various amounts of Ca^{2+}. The concentration of free Ca^{2+} at pH 7.2 was calculated by means of a computer program (Fabiato & Fabiato, 1979) which corrected for the concentrations of K^+, Mg^{2+}, and ATP present in the assay. Ca^{2+} transport was initiated by the addition of crude extracts or Percoll/KCl gradient fractions to assay mixtures preincubated at 22 °C for 1 min. Membrane preparations (approx. 2 mg of protein in 200–400 μl) were added to a final assay volume of 1100 μl. At specific times, 200 μl aliquots were withdrawn and pipetted into 1 ml of ice-cold termination buffer (20 mM-Hepes/NaOH/6% sucrose/5 mM-$MgCl_2$/225 mM-$CaCl_2$, pH 7.2) and 1 ml samples were rapidly filtered through 0.45 μm HAWP 025 Millipore filters pre-rinsed with 2 ml of wash buffer (20 mM-Hepes/NaOH/6% sucrose/5 mM-$MgCl_2$/1 mM-$CaCl_2$, pH 7.2). Filters were washed with two 4 ml portions of wash buffer, solubilized in 1 ml of 2-ethoxyethanol and counted in 3.5 ml of an aqueous scintillant, Permafluor 2 (Packard). ATP-independent $^{45}Ca^{2+}$ uptake was assessed by pre-incubating membrane samples on ice for 5 min with 1 mg of apyrase/ml. Non-specific $^{45}Ca^{2+}$ binding was determined in assay tubes containing a 2000-fold excess of $CaCl_2$. Terminated samples were filtered within 10 min, since radioactivity loss (up to 5% in 10 min) sometimes occurred when $^{45}Ca^{2+}$-loaded membrane vesicles were left in termination buffer for prolonged periods.

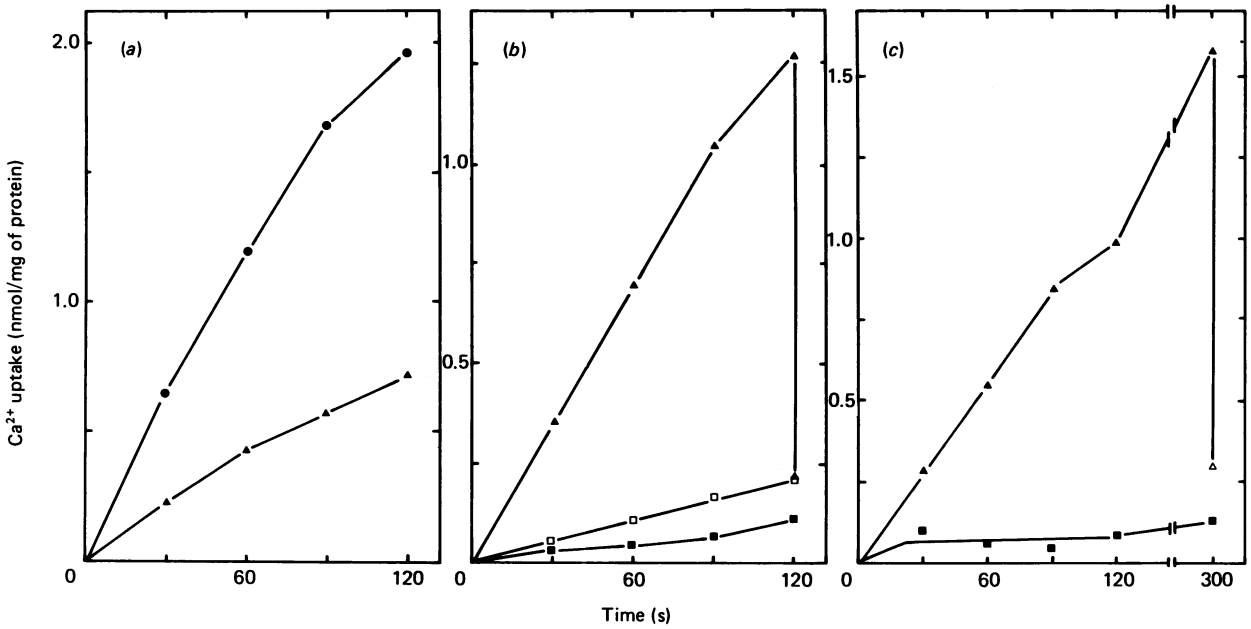

Fig. 1. Time course of Ca^{2+} accumulation by crude cell homogenates and isolated membrane vesicles

(*a*) Crude sonicates of 2 h starved cells were prepared and assayed for Ca^{2+} uptake in the absence (●) or presence (▲) of mitochondrial inhibitors as described by Mato & Marín-Cao (1979). (*b*) Crude homogenates, prepared as in (*a*), were assayed for Ca^{2+} uptake in the presence of mitochondrial inhibitors under the conditions of Mato & Marín-Cao (▲) or under the same conditions without added Mg^{2+} (□). After 2 min, a sample was removed from the complete system, added to termination buffer containing 25 μM-A23187 and filtered (△). Cell extract was also preincubated with apyrase and assayed without added ATP (■). (*c*) Fractions 4 and 5 from Percoll/KCl gradients of sonicated cell extracts were pooled and assayed for Ca^{2+} uptake under standard conditions (see the Experimental section) (▲). After 5 min, a sample was transferred to termination buffer containing A23187 (△). Membranes were also preincubated with apyrase and assayed for Ca^{2+} uptake in the absence of added ATP (■). Since the magnitude of Ca^{2+} uptake varied between preparations, a representative experiment is shown in each panel; however, qualitatively similar results were obtained in at least two additional experiments of each type.

Other enzyme assays

α-Glucosidase-2 activity was assayed according to Borts & Dimond (1981) except that a 40 mM-Tris/HCl buffer, pH 7.5, was used. Alkaline phosphatase was measured as described by Lee *et al.* (1975). Succinate dehydrogenase activity was determined using the procedure of Lee *et al.* (1975) with slight modifications. Briefly, 200 μl aliquots of Percoll/KCl gradient fractions were added to 1.8 ml assay mixtures containing 37.5 mM-K_2HPO_4, 20 mM-NaN_3, 30 mM-EGTA and 0.3 mM-dichlorophenolindophenol, pH 7.6. Following incubation at 22 °C for 15 min, succinate dehydrogenase activity was measured by adding 200 μl of 1 M-sodium succinate/NaOH, pH 7.6, to the assay mixture and monitoring the decrease in absorbance at 600 nm using a Pye–Unicam model SP8-500 recording spectrophotometer. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

RESULTS

Ca^{2+}-transporting activity in crude cell extracts

As reported by Mato & Marín-Cao (1979), crude cell sonicates of *D. discoideum* in the presence of ATP rapidly accumulated Ca^{2+} into structures detectable by a filtration assay. Addition of the mitochondrial inhibitors NaN_3 (100 μM) and Ruthenium Red (12 μM) and the uncouplers dinitrophenol (100 μM) and CCCP (10 μM) inhibited Ca^{2+} uptake by 60–70 % (Fig. 1*a*). These results suggest the presence of at least two Ca^{2+}-transporting systems in crude homogenates of these cells.

Non-mitochondrial Ca^{2+} accumulation was ATP- and Mg^{2+}-dependent (Fig. 1*b*). In the absence of apyrase (1 mg/ml), ATP-independent Ca^{2+} uptake was about 2-fold higher. Ca^{2+} transport into non-mitochochondrial stores normally proceeded linearly for 1.5–2 min. Inclusion of an ATP-regenerating system did not enhance Ca^{2+} uptake; therefore, ATP depletion was not the rate-limiting step in this process. Also, little of the accumulated vesicular Ca^{2+} appeared to be membrane-bound or precipitated, since more than 80 % of this Ca^{2+} was readily released by the addition of the Ca^{2+} ionophore A23187 (Fig. 1*b*).

Isolation of a non-mitochondrial Ca^{2+} pump

To isolate the non-mitochondrial ATP/Mg^{2+}-dependent Ca^{2+} pump, sonicated cell homogenates were fractionated on Percoll/KCl gradients. The distribution of membrane marker enzymes in this gradient is illustrated in Fig. 2(*a*). Under these fractionation conditions, peak Ca^{2+}-accumulating activity typically appeared in fraction 4, the top, visible membrane band of the gradient. Alkaline phosphatase, an enzyme primarily associated with the external surface of the plasma membrane of *Dictyostelium* (Glomp *et al.*, 1985) also exhibited maximum specific activity in this fraction. However, compared with the profile of Ca^{2+} uptake, the

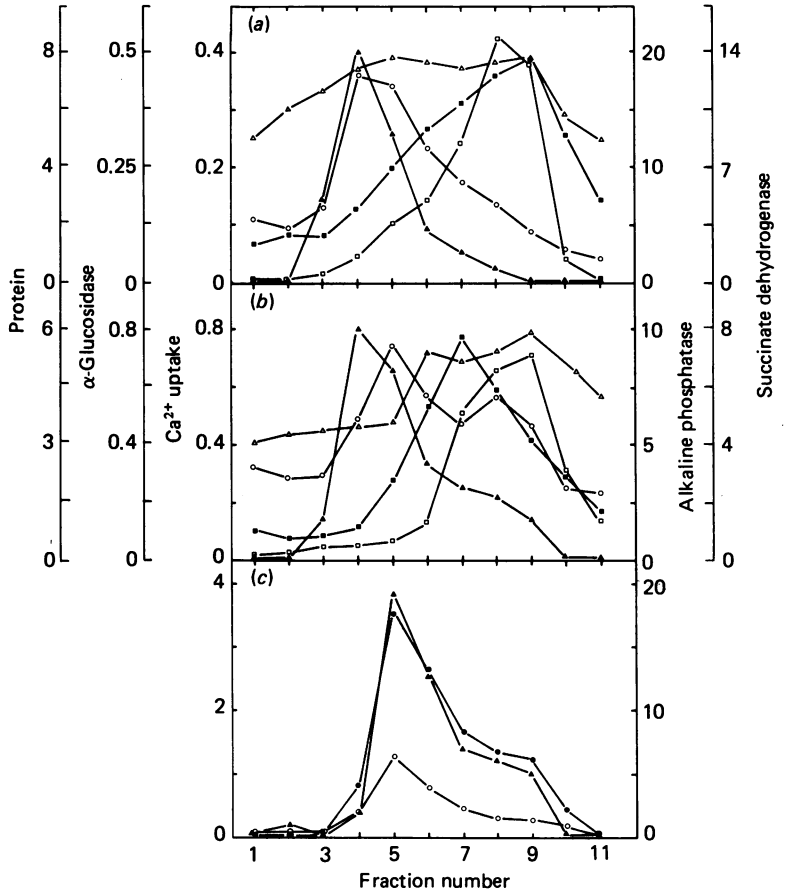

Fig. 2. Fractionation of crude cell homogenates on Percoll/KCl gradients

Cell lysates were prepared by (*a*) sonication or by (*b*) filter breakage and fractionated on Percoll/KCl gradients. Fractions were assayed for ATP-dependent Ca^{2+} uptake (▲), alkaline phosphatase (○), α-glucosidase-2 (■) and succinate dehydrogenase (□) activities and protein concentration (△). All enzyme activities are expressed as nmol/min per mg of protein; protein concentration is given in mg/ml. (*c*) Fractions 4 and 5 from Percoll/KCl gradients of filter-broken cells were pooled, diluted to 12 ml with 20 mM-Hepes, pH 7.2, and centrifuged at 40000 *g* for 30 min. The loose membrane pellet was withdrawn, resuspended in 3 ml of 20 mM-Hepes and recentrifuged through a second Percoll/KCl gradient. Fractions were assayed for 2 min for ATP-dependent Ca^{2+} uptake (▲) and for 20 min for alkaline phosphatase activity in the absence (○) and presence (●) of 0.2% Triton X-100. The experiments in each panel were repeated at least once with similar results.

alkaline phosphatase activity was skewed towards higher density fractions. Succinate dehydrogenase, a mitochondrial inner membrane marker (Schneider, 1946), and α-glucosidase-2, an enzyme reported to be associated with rough endoplasmic reticulum in *Dictyostelium* (Mierendorf *et al.*, 1985), both displayed low specific activity in fraction 4. These two activities were consistently found near the bottom of the gradient in fractions 8 and 9. While these results suggest that the Ca^{2+} pump is not localized in the mitochondrion or in the rough endoplasmic reticulum, they do not provide conclusive evidence that the enzyme is associated with the plasma membrane.

Similar membrane marker profiles were obtained when filter-broken cell extracts were fractionated on Percoll/KCl gradients, except that alkaline phosphatase activity was higher in fractions 5–9 of the gradient (Fig. 2*b*). Maximum Ca^{2+}-accumulating activity in these extracts was always observed in gradient fractions 4 or 5. When membranes isolated from these fractions were recentrifuged through a second Percoll/KCl gradient, and the resultant fractions were assayed for Ca^{2+}-accumulating and alkaline phosphatase activities, the two activities were observed to cosediment. Moreover, the alkaline phosphatase activity associated with these membranes was increased dramatically in the presence of 0.2% Triton X-100 (Fig. 2*c*). These results suggest that the Ca^{2+} pump is probably associated with 'inside-out' plasma membrane vesicles.

Characterization of the isolated Ca^{2+} pump

Since Ca^{2+}-pumping activity could be effectively and reproducibly separated from mitochondria and rough endoplasmic reticulum using Percoll/KCl gradients, membranes from fractions 4 and 5 of these gradients (fraction 4/5 membranes) were used to characterize the transport enzyme. Initial experiments were performed with fractionated membranes of sonicated cell homogenates, while later studies used membranes of filter-broken extracts (see under 'Experimental'). Results obtained with the two membrane preparations were comparable. The breakage method employed in each experiment is indicated in the Figure legends or in the text.

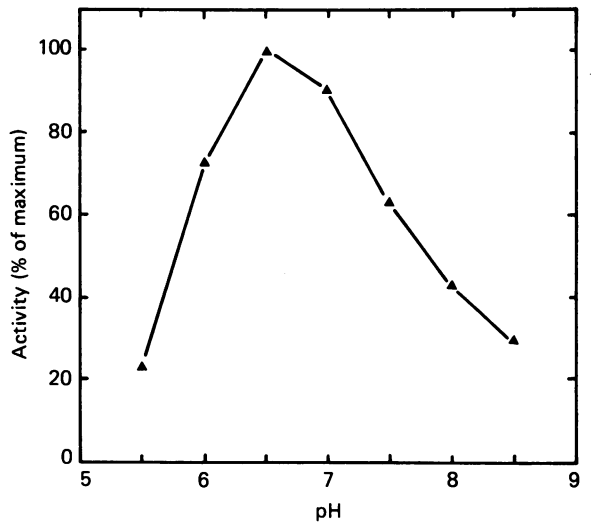

Fig. 3. Effect of pH on Ca^{2+} uptake by isolated membrane vesicles

Cell homogenates prepared by filter-breakage were fractionated on Percoll/KCl gradients as described in the Experimental section, except that the gradient buffer contained 5 mM-Hepes, pH 7.2. Fraction 4/5 membrane vesicles were assayed for 2 min for ATP-dependent Ca^{2+} transport in a modified uptake medium containing 10 μM-$CaCl_2$ (no EGTA) and 65 mM-Tris/malate buffer adjusted to the indicated pH. Each point represents the average of duplicate determinations in a single experiment. Similar results were obtained in three additional experiments.

As in crude extracts, ATP-dependent Ca^{2+} accumulation by the isolated vesicles progressed linearly for about 2 min and uptake normally continued for 5–6 min. Upon addition of A23187, over 80% of the sequestered $^{45}Ca^{2+}$ was released (Fig. 1*c*). Mg^{2+}-depleted fraction 4/5 membranes required 5 mM-Mg^{2+} for maximum Ca^{2+} accumulation (results not shown). Changes in the extravesicular pH markedly affected Ca^{2+}-pumping activity; Ca^{2+} transport increased sharply from pH 5.5 to the pH optimum of 6.5–7.0, and little activity was detectable above pH 8.5 (Fig. 3). The Ca^{2+} pump exhibited high specificity for ATP as the energy source (Table 1). The rate of Ca^{2+} uptake in the presence of ITP was only 16% of the rate with ATP, and the other compounds tested were even less effective.

To determine the effect of Ca^{2+} concentration on pump activity, initial reaction velocities were determined at free extravesicular Ca^{2+} concentrations ranging from 0.05 to 2 μM. The results, plotted according to Hanes (1932), are presented in Fig. 4. Over this concentration range of free Ca^{2+}, the enzyme displayed Michaelis–Menten kinetics with an apparent K_m of 0.3 μM and a $V_{max.}$ of 3 nmol of Ca^{2+} transported/min per mg of protein.

The following compounds, at the concentrations indicated, had no detectable effect on Ca^{2+} accumulation by membrane vesicles isolated from filter-broken cell extracts: 30 μM-Ruthenium Red, 100 μM-ouabain, 100 μM-verapamil and 150 μM-diltiazem. Vanadate was a potent inhibitor of Ca^{2+} transport with an IC_{50} of approx. 2 μM (Fig. 5).

Calmodulin has been reported to activate plasma membrane Ca^{2+}-transport ATPases from a variety of cell types (Penniston, 1983). To determine if the Ca^{2+} pump isolated in this study is also regulated by calmodulin, initial rates of Ca^{2+} transport were measured in the absence and presence of different concentrations of the calmodulin antagonist TFP. At a concentration of 10 μM, TFP inhibited Ca^{2+} accumulation by crude homogenates (seven experiments) and fractionated membrane vesicles prepared by sonication (five experiments) by less than 16%. When the concentration of this drug was increased to 100 μM, Ca^{2+}-transporting activity was reduced by as much as 65% in certain experiments;

Table 1. Utilization of various nucleoside triphosphates and *p*-nitrophenylphosphate by the isolated Ca^{2+} pump

Fraction 4/5 membranes from Percoll/KCl gradients of sonicated cell extracts were assayed for Ca^{2+} uptake for 2 min as described in the Experimental section, except that the 2 mM-ATP was replaced by other possible substrates at the same concentration. Results are expressed relative to ATP and are average values ± S.E.M. for three experiments performed in duplicate.

Compound	Ca^{2+} uptake (%)
ATP	100
ITP	16.6 ± 3.8
UTP	8.6 ± 5.3
GTP	3.9 ± 3.5
CTP	2.9 ± 2.8
pNPP	0

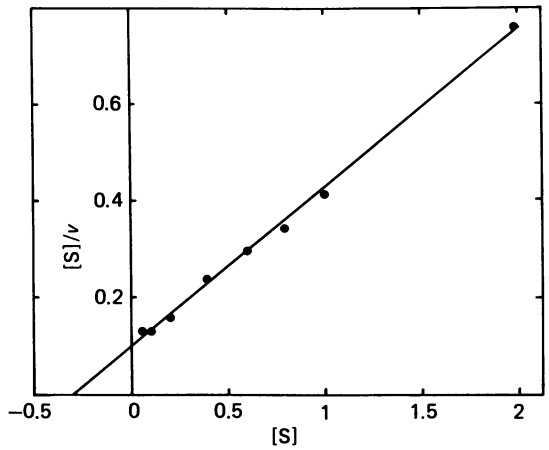

Fig. 4. Hanes–Woolf plot of initial rates of Ca^{2+} uptake as a function of free extravesicular Ca^{2+} concentration

Fraction 4/5 membranes from Percoll/KCl gradients of filter-broken cell extracts were assayed for ATP-dependent Ca^{2+} uptake under standard conditions, except the free Ca^{2+} concentration was varied from 0.05 to 2.0 μM. To determine initial rates of Ca^{2+} uptake, samples were terminated 20, 40, and 60 s after the start of the reaction. [S] is the micromolar concentration of free Ca^{2+} and v is the initial rate of Ca^{2+} uptake expressed as nmol/min per mg of protein. The intersect of the line with the x-axis is equal to $-K_m$. The line was fitted by regression analysis. Each point represents the average of data from three independent experiments.

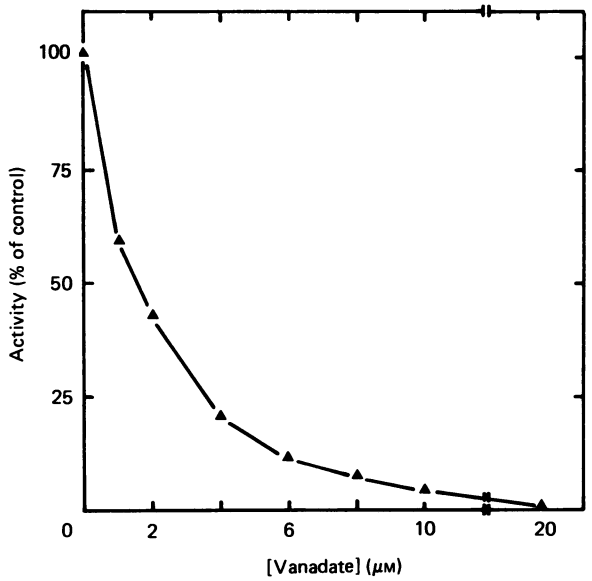

Fig. 5. Effect of vanadate on Ca^{2+} uptake by isolated membrane vesicles

Fraction 4/5 membranes from Percoll/KCl gradients of filter-broken cell extracts were incubated on ice for 5 min with increasing concentrations of vanadate and then assayed for 2 min for ATP-dependent Ca^{2+} uptake. Each point represents the average of duplicate determinations in a single experiment. Similar results were obtained in two additional experiments.

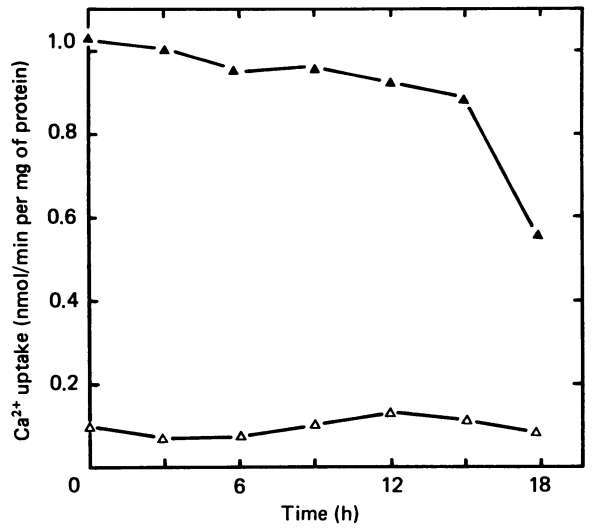

Fig. 6. Development profile of non-mitochondrial Ca^{2+}-accumulating activity in crude homogenates

Amoebae were grown, washed, and permitted to develop on phosphate-buffered agar as described by Coukell *et al.* (1984). At the times indicated, 5×10^8 cells were harvested, washed twice in 20 mM-Hepes, pH 7.2, resuspended to 2×10^8 cells/ml in the same buffer, and lysed by the filter-breakage method. Samples were preincubated on ice for 5 min in the absence (▲) or presence (△) of 5 μM-vanadate, and then assayed for 2 min in duplicate for ATP-dependent Ca^{2+} uptake. Results are an average of data from three independent experiments.

however, the degree of inhibition varied considerably (24–65%). Similarly, another calmodulin antagonist, calmidazolium, at 10 μM, failed to decrease the Ca^{2+}-pumping activity of filter-broken cell extracts by more than 7% (two experiments). Ca^{2+}-transporting activity was also unaffected by the addition of 7 μg of bovine calmodulin/ml and 20 μM-Ca^{2+} to fractionated membranes washed extensively with HAMK buffer containing 2 mM-EGTA to remove endogenous calmodulin (two experiments) (results not shown).

IP_3 releases Ca^{2+} from intracellular, non-mitochondrial stores of a wide variety of cells, including *D. discoideum* (Europe-Finner & Newell, 1986*b*), and GTP appears to exert a similar effect on certain cell types (Gill *et al.*, 1986). Therefore, these compounds were examined for the ability to promote Ca^{2+} efflux from membrane vesicles isolated from sonicated cell homogenates. Membrane vesicles were preloaded with $^{45}Ca^{2+}$ for 6 min and then incubated for 1 min with either 25 μM-IP_3, 20 μM-GTP, both IP_3 and GTP, or an equivalent volume of water. In four independent experiments, these compounds failed to induce a detectable Ca^{2+} efflux despite the fact that A23187 released at least 75% of the accumulated Ca^{2+} from the vesicles (results not shown).

Ca^{2+}-transporting activity during development

To determine if the non-mitochondrial Ca^{2+}-pumping activity changes appreciably during development, ATP-dependent Ca^{2+} uptake was measured in crude homogenates of cells permitted to develop on phosphate-buffered agar. As shown in Fig. 6, Ca^{2+}-transporting activity was essentially constant during the first 15 h of development. In all three experiments, however, the specific activity declined significantly between 15 and 18 h of development. Unfortunately, later stages of development could not be assayed due to difficulties in preparing lysates by the filter-breakage technique. At all time points, Ca^{2+}-transporting activity was inhibited more than 85% by 5 μM-vanadate; this degree of inhibition was similar to the effect of vanadate on the isolated Ca^{2+} pump of 2 h starved cells (compare Figs. 5 and 6). Therefore, the high-affinity non-mitochondrial Ca^{2+}-transporting activity measured in crude cell extracts during development could be associated with the Ca^{2+} pump isolated on Percoll/KCl gradients.

DISCUSSION

To investigate the mechanisms regulating intracellular Ca^{2+} homeostasis in *D. discoideum*, we have characterized a high-affinity Ca^{2+}-transport system associated with the plasma membrane of this organism. In this study, ATP-dependent $^{45}Ca^{2+}$ uptake into membrane vesicles was monitored rather than Ca^{2+}-dependent ATPase activity because high-affinity Ca^{2+}-ATPases not associated with Ca^{2+} transport have been reported in plasma membranes of rat liver (Lin, 1985) and rat corpus luteum (Minami & Penniston, 1987). Furthermore, plasma membranes of *D. discoideum* possess several Mg^{2+}-dependent ATPase activities (MacDonald & Weeks, 1984; Pogge-von Strandmann *et al.*, 1984; Serrano *et al.*, 1985) and a ouabain-insensitive, monovalent cation-stimulated ATPase (Blanco, 1982) which could obscure detection of high-affinity Ca^{2+}-ATPase activity. Since the Ca^{2+}-transport system described in this paper is ATP-

dependent (Fig. 1), has a high affinity for Ca^{2+} (Fig. 4) and is not inhibited by Ca^{2+}-channel blockers, it is probably a Ca^{2+} pump and not a channel or the low-affinity Ca^{2+} carrier described by Europe-Finner & Newell (1985).

Localization of the isolated Ca^{2+} pump to the plasma membrane is supported by the following observations. First, Percoll/KCl gradient fractions possessing peak Ca^{2+}-accumulating activity were not contaminated appreciably by mitochondrial or rough endoplasmic reticulum membrane markers (Figs. 2*a* and 2*b*). Second, when the Ca^{2+} transporter was isolated and recentrifuged through a second Percoll/KCl gradient, it cosedimented with alkaline phosphatase activity (Fig. 2*c*), a plasma membrane marker (Gilkes & Weeks, 1977; Glomp *et al.*, 1985). When crude extracts were run on these gradients, the alkaline phosphatase activity was often distributed broadly through the gradient and the Ca^{2+}-accumulating activity was associated predominantly with the low-density fractions possessing phosphatase activity (Figs. 2*a* and 2*b*). These results suggest that the Ca^{2+}-transport system resides in a subpopulation of plasma membrane vesicles. In an earlier study, Das & Henderson (1983) observed that fractionation of filter-broken *D. discoideum* extracts on sucrose gradients resolved plasma membranes into three discrete bands with very similar protein composition. However, since the relative amounts of certain proteins (such as actin) varied, the authors concluded that the migration of plasma membranes was probably affected by the degree of cytoskeletal association with the membranes or by different protein-to-lipid ratios rather than by membrane heterogeneity caused by fusion of different classes of membranes. Also, Goodloe-Holland & Luna (1986) observed that sealed *Dictyostelium* plasma membrane vesicles sediment at lower density on Dextran T-70 gradients than do unsealed plasma membrane vesicles or fragments. Since the alkaline phosphatase activity of the Ca^{2+}-accumulating vesicles was elevated greatly in the presence of a detergent (Fig. 2*c*), these findings, taken together, suggest that the Ca^{2+} pump is probably a component of 'inside-out' plasma membrane vesicles. Third, the Ca^{2+} pump isolated in this study has properties closer to those of plasma membrane Ca^{2+} pumps of other cell types (reviewed in Rega & Garrahan, 1986) than intracellular Ca^{2+} pumps. For example, like other plasma membrane pumps, the *Dictyostelium* pump exhibited a marked preference for ATP as an energy-donating substrate during active Ca^{2+} transport (Table 1). In contrast, the Ca^{2+} pump of the sarcoplasmic reticulum utilizes other ribonucleotide triphosphates and pNPP (de Meis, 1980). Also, the Ca^{2+} pumps of the sarcoplasmic reticulum (Caroni & Carafoli, 1981) and neutrophil endoplasmic reticulum (Prentki *et al.*, 1984) are considerably less sensitive to inhibition by vanadate than is the pump characterized in the present study (Fig. 5) and plasma membrane pumps (Rega & Garrahan, 1986). Finally, addition of IP_3 and/or GTP to the isolated $^{45}Ca^{2+}$-loaded membrane vesicles failed to promote Ca^{2+} efflux despite the fact that IP_3 has been reported to induce release of Ca^{2+} from intracellular non-mitochondrial stores in *Dictyostelium* (Europe-Finner & Newell, 1986*b*).

To determine if the Ca^{2+} pump is regulated by calmodulin, both pharmacological and calmodulin-supplementation experiments were performed. Interestingly, neither TFP nor calmidazolium, at concentrations where they have been reported to act as specific calmodulin antagonists (Roufogalis, 1982; Gietzen *et al.*, 1981), had a significant effect on Ca^{2+}-pumping activity. At higher concentrations (e.g. 100 μM), TFP partially inhibited Ca^{2+} transport but the results were extremely variable; thus non-specific drug effects (Roufogalis, 1982) cannot be discounted. In addition, supplementation of isolated EGTA-treated membrane vesicles with exogenous calmodulin did not affect ATP-dependent Ca^{2+} uptake. While these results suggest that this Ca^{2+} pump of *Dictyostelium* is not regulated by calmodulin, studies in other systems have shown that it is often difficult to demonstrate an interaction between calmodulin and transport ATPases (Rega & Garrahan, 1986). Therefore, the possibility that this pump is regulated by calmodulin cannot be ruled out at this time.

What is the probable function of this Ca^{2+} pump in *Dictyostelium*? Results from the present study together with earlier findings (Mato & Marín-Cao, 1979; Europe-Finner & Newell, 1986*b*) suggest that at least three potential Ca^{2+}-regulating systems exist in this organism: a low-affinity mitochondrial transporter, a high-affinity non-mitochondrial intracellular pump, and the high-affinity plasma membrane pump. During cyclic AMP-mediated cell stimulation, the concentration of total cellular Ca^{2+} is estimated to increase by 60–100 μM (Wick *et al.*, 1978; Bumann *et al.*, 1984), and this is probably accompanied by an increase in free cytosolic Ca^{2+}. Restoration of free cytosolic Ca^{2+} to low micromolar levels is likely performed primarily by the low-affinity high-capacity mitochondrial system. Further reduction to nanomolar concentrations probably involves the concerted action of the two high-affinity pumps. However, since intracellular stores are of limited capacity, the plasma membrane pump probably plays the major role in re-establishing the resting concentration of free intracellular Ca^{2+}. This proposal is supported by the observation that the K_m for Ca^{2+} transport by this system is 0.3 μM, the estimated cytosolic free Ca^{2+} concentration of these cells (Bumann *et al.*, 1984). A 'house-keeping' function for this pump is also suggested by the observation that its activity appears to be relatively constant during at least the first 15 h of development (Fig. 6).

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