Uptake of Ca^{2+} Driven by the Membrane Potential in Energy-depleted Yeast Cells

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(Received 10 June 1986; revised 16 January 1987)

The time-course of $^{45}\text{Ca}^{2+}$ influx into yeast cells was measured under non-steady-state conditions obtained by preincubating the cells in a Ca^{2+} -free medium containing glucose and buffer. Two components were distinguished: a saturable component which reached a steady-state after about 40 s of $^{45}\text{Ca}^{2+}$ uptake and a linear increase in cellular $^{45}\text{Ca}^{2+}$ starting after 60–90 s. Using differential extraction methods it was determined that after 20 s of uptake, $^{45}\text{Ca}^{2+}$ was localized in the cytoplasmic pool and in bound form with no $^{45}\text{Ca}^{2+}$ in the vacuole. After 3 min most of the cellular $^{45}\text{Ca}^{2+}$ was concentrated in the vacuole and in bound form. The initial rate of $^{45}\text{Ca}^{2+}$ uptake under non-steady-state conditions thus measured $^{45}\text{Ca}^{2+}$ transport across the plasma membrane without interference by vacuolar uptake. The effect of membrane potential ($\Delta\psi$) on this transport was investigated in cells depleted of ATP. A high $\Delta\psi$ was produced by preincubating the cells with trifluoperazine (TFP) and subsequently washing the cells free from TFP. Substantial $^{45}\text{Ca}^{2+}$ influx was measured in the absence of metabolic energy in cells with a high $\Delta\psi$. Below a threshold value of -69.5 mV the logarithms of the initial rate of $^{45}\text{Ca}^{2+}$ influx and of the steady-state level of the first component were linear with respect to $\Delta\psi$. It is suggested that $^{45}\text{Ca}^{2+}$ influx across the plasma membrane is mediated by channels which open when $\Delta\psi$ is below a threshold value. The results indicated that Ca^{2+} influx across the plasma membrane was driven electrophoretically by $\Delta\psi$.

INTRODUCTION

Cytoplasmic Ca^{2+} homeostasis is essential for the regulatory function of Ca^{2+} ions in eukaryotic cells. In yeast cells, cytoplasmic Ca^{2+} homeostasis is maintained by the vacuolar Ca^{2+} uptake system (Eilam *et al.*, 1985a) which transports Ca^{2+} against its electrochemical gradient. The energy for this transport is derived from the $\Delta\bar{\mu}_{\text{H}^+}$ across the vacuolar membrane (Ohsumi & Anraku, 1983), which is maintained by the vacuolar H^+ -ATPase (Kakinuma *et al.*, 1981). The properties of the vacuolar Ca^{2+} transport system have been investigated in vacuolar membrane vesicles (Ohsumi & Anraku, 1983), and in permeabilized cells (Eilam *et al.*, 1985a). Less information is available on the properties of plasma membrane Ca^{2+} transport system(s) since compartmentalization of Ca^{2+} within the cells makes it difficult to distinguish between transport across the plasma membrane and across the vacuolar membrane. Ca^{2+} uptake in yeasts is an energy-requiring process (Borst-Pauwels, 1981) but it is not clear whether energy is required for the initial step of Ca^{2+} transport across the plasma membrane or for subsequent accumulation into the vacuole. The mechanism of coupling between transport across the plasma membrane and energy supply is not yet clear. In the present work we distinguish between transport across plasma and vacuolar membranes by measurements under non-steady-state conditions. A preliminary report of this work has been presented (Eilam *et al.*, 1985b).

Abbreviations: TPP⁺, tetraphenylphosphonium ion; TFP, trifluoperazine; $\Delta\bar{\mu}_{\text{H}^+}$, transmembrane proton electrochemical gradient; $\Delta\psi$, membrane potential.

METHODS

Organism and culture conditions. *Saccharomyces cerevisiae* strain N123 (genotype *MATa/α his1*) was maintained at 4 °C on YPD-agar slopes and grown at 30 °C in YPD-broth (Bacto yeast extract 10 g l⁻¹, Bacto peptone 20 g l⁻¹, glucose 20 g l⁻¹). Cells were collected from an overnight culture by centrifugation, washed three times by resuspension in distilled water and finally resuspended in the indicated medium at a cell density of 5×10^7 cells ml⁻¹.

Preparation of cells with different $\Delta\psi$ values. Non-steady-state conditions for $^{45}\text{Ca}^{2+}$ uptake measurements were achieved by preincubating the cells for 90 min at 30 °C with shaking in medium containing MES/Tris (10 mM, pH 6.0) and glucose (100 mM) (buffer-glucose medium). Following this preincubation cells were further preincubated for 30 min at 30 °C in buffer-glucose medium which contained concentrations of TFP between 0 and 30 μM in order to obtain cells with different $\Delta\psi$ values. Cells were then collected by centrifugation, washed once with MES/Tris buffer (10 mM, pH 6.0) and finally resuspended at 10^8 cells ml⁻¹ in buffer which contained antimycin A (15 μM) and 2-deoxyglucose (5 mM). After 5 min in this medium, transport measurements were initiated. In some experiments cells with different $\Delta\psi$ values were obtained by preincubating as above with 40 μM -TFP and then centrifuging and washing in buffer. Before the initiation of the transport, the cells were preincubated in buffer for different time intervals between 10 and 60 min. During this preincubation the $\Delta\psi$ was partially dissipated as a function of the length of the preincubation. We did not see any effect of the way the specific value of $\Delta\psi$ had been obtained on Ca^{2+} influx.

Measurements of Ca^{2+} influx. Ca^{2+} influx was initiated by the addition of 0.5 ml of cells suspended in buffer (10^8 cells ml⁻¹) to 0.5 ml buffer (MES/Tris; 10 mM, pH 6.0) containing Ca^{2+} (2×10^{-6} M) labelled with $^{45}\text{Ca}^{2+}$ [1 $\mu\text{Ci ml}^{-1}$ (37 kBq ml⁻¹)], antimycin A and deoxyglucose as above. Transport was measured at 25 or 30 °C as indicated. The influx was terminated by the addition of LaCl_3 (0.1 mM) followed by 1 ml washing solution containing MgCl_2 (20 mM) and LaCl_3 (0.1 mM); the cells were then filtered immediately on glass fibre filters and washed five times with 2 ml portions of the washing solution. The filters were dried and the radioactivity was determined after the addition of toluene-based scintillation fluid using a liquid scintillation counter. Ca^{2+} binding was determined by 'zero-time' measurements, which were obtained by the addition of 0.5 ml cell suspension to 0.5 ml transport solution containing Ca^{2+} (2×10^{-6} M) labelled with $^{45}\text{Ca}^{2+}$, plus LaCl_3 (0.2 mM). The cells were filtered immediately as described above.

Ca^{2+} uptake in the absence of an induced $\Delta\psi$ was measured in cells preincubated as above but without TFP. In some experiments glucose (100 mM) was included in the transport medium and antimycin A and deoxyglucose were omitted as indicated.

$\Delta\psi$ measurements. These were based on the steady-state distribution of [^3H]TPP⁺ between cells and medium. $\Delta\psi$ and Ca^{2+} uptake were determined in the same cells by double-labelling with [^3H]TPP⁺ and $^{45}\text{Ca}^{2+}$. [^3H]TPP⁺ [1 μM ; 0.05 $\mu\text{Ci ml}^{-1}$ (1.85 kBq ml⁻¹)] was included in all preincubation and transport media at the same concentration and specific activity, to allow enough time for [^3H]TPP⁺ penetration into the cells. The results were corrected for the binding of [^3H]TPP⁺. Cell volume was determined and $\Delta\psi$ values were calculated as described by Eilam *et al.* (1985c).

Differential extraction of $^{45}\text{Ca}^{2+}$ from cytosol and vacuole after $^{45}\text{Ca}^{2+}$ influx. After preincubation in buffer-glucose medium to obtain non-steady-state conditions, transport was measured for 0, 20 or 180 s. Each transport measurement was repeated nine times; after termination of transport by addition of 0.1 mM- LaCl_3 the cells were filtered on a membrane filter and washed five times with washing solution at 0 °C and once with ice-cold buffered sorbitol (10 mM-MES/Tris, pH 6.0–0.7 M-sorbitol). Three of these filters, which served to determine the total amount of $^{45}\text{Ca}^{2+}$ in the cells, were dried and the radioactivity was determined after this filtration. Three additional filters were removed and immersed in flasks containing 3 ml buffered sorbitol and DEAE-dextran (0.56 mg ml⁻¹) at 0 °C. The cells were resuspended by shaking in a vortex shaker and incubated at 0 °C for 30 s. The cells were then filtered on membrane filters and washed four times with buffered sorbitol at 0 °C. Each filter was treated separately and the extraction was done immediately after Ca^{2+} uptake. The filters were dried and the radioactivity was determined. This procedure yielded the sum of bound $^{45}\text{Ca}^{2+}$ and vacuolar $^{45}\text{Ca}^{2+}$. Cytoplasmic $^{45}\text{Ca}^{2+}$ was determined from the difference between total cellular Ca^{2+} and this determination. Three additional filters, containing cells which were washed with washing solution and buffered sorbitol, were extracted to determine the bound Ca^{2+} . The filters were immersed in 3 ml distilled water. The cells were resuspended and methanol (4 ml) was added. The suspensions were then incubated for 10 min at room temperature to release the unbound ions. The cells were collected by filtration, washed once with 60% (v/v) methanol and three times with water. The filters were dried and the radioactivity, which represents the bound $^{45}\text{Ca}^{2+}$, was determined. The details of the procedure of differential extraction were given by Eilam *et al.* (1985a).

RESULTS

The time-course of $^{45}\text{Ca}^{2+}$ influx into yeast cells was measured under two sets of conditions.

Fig. 1

Fig. 2

Fig. 1. Time-course of $^{45}\text{Ca}^{2+}$ influx into yeast cells under steady-state and non-steady-state conditions. Yeast cells were preincubated at 10^8 cells ml^{-1} in medium containing MES/Tris buffer (pH 6.0, 10 mM) and glucose (100 mM). To obtain steady-state conditions CaCl_2 (10^{-6} M) was included in the medium (buffer-glucose- Ca^{2+} medium) and the cells were preincubated for 3 h at 30 °C. Transport was initiated by the addition of 0.5 ml cell suspension to 0.5 ml buffer-glucose- Ca^{2+} medium labelled with $^{45}\text{Ca}^{2+}$ [$1 \mu\text{Ci ml}^{-1}$ (37 kBq ml^{-1})]. To obtain non-steady-state conditions the cells were preincubated as above but without CaCl_2 for 90 min at 30 °C. Transport was initiated by the addition of 0.5 ml cell suspension to 0.5 ml buffer-glucose medium containing CaCl_2 (2×10^{-6} M) labelled with $^{45}\text{Ca}^{2+}$ [$1 \mu\text{Ci ml}^{-1}$ (37 kBq ml^{-1})]. Transport was measured at 30 °C. $\blacktriangle$, Steady-state conditions; $\bullet$, non-steady-state conditions.

Fig. 2. Effects of glucose, Zn^{2+} , La^{3+} and A23187 on the time-course of Ca^{2+} influx under non-steady-state conditions. $^{45}\text{Ca}^{2+}$ influx was measured at 30 °C under non-steady-state conditions as described in Methods in medium containing MES/Tris (10 mM, pH 6.0), CaCl_2 ($1 \mu\text{M}$) labelled with $^{45}\text{Ca}^{2+}$ [$0.5 \mu\text{Ci ml}^{-1}$ (18.5 kBq ml^{-1})] and the following: 100 mM-glucose ($\circ$); 100 mM-glucose + $0.1 \mu\text{M}$ -A23187 ($\bullet$); 100 mM-glucose + 0.1 mM - ZnCl_2 ($\square$); 100 mM-glucose + 0.1 mM - LaCl_3 ($\blacktriangle$) or no additions ($\triangle$).

(1) Steady-state distribution of Ca^{2+} between intracellular water and the medium was achieved by preincubating the cells with buffer-glucose medium and Ca^{2+} (10^{-6} M) for 3 h. $^{45}\text{Ca}^{2+}$ influx was initiated by the addition of $^{45}\text{Ca}^{2+}$ without added Ca^{2+} . Under these conditions $^{45}\text{Ca}^{2+}$ influx represents $\text{Ca}^{2+}/^{45}\text{Ca}^{2+}$ exchange (Fig. 1).

(2) Non-steady-state kinetics were obtained by preincubating the cells with buffer-glucose medium without added Ca^{2+} . Uptake was initiated by the addition of Ca^{2+} (10^{-6} M) labelled with $^{45}\text{Ca}^{2+}$. The time-course of Ca^{2+} uptake under these conditions had two phases (Fig. 1). The first was a fast influx component which became saturated after 30–40 s; the second was a linear increase in cellular $^{45}\text{Ca}^{2+}$ which followed the first component after 60–90 s. Curves of the same general shape were obtained in 56 different experiments.

In order to determine whether the first component of Ca^{2+} influx under non-steady-state conditions represented only $^{45}\text{Ca}^{2+}$ binding or, in addition, transport across the plasma membrane, we added the Ca^{2+} ionophore A23187 together with $^{45}\text{Ca}^{2+}$. The Ca^{2+} ionophore caused an increase in the rate and the extent of Ca^{2+} influx during the first phase (Fig. 2), indicating that the first component represents transport across membranes in addition to Ca^{2+} binding.

The amounts of bound $^{45}\text{Ca}^{2+}$ were determined by several methods. (a) La^{3+} ions were added before the addition of $^{45}\text{Ca}^{2+}$; the cell associated counts represented the 'zero-time' value of Ca^{2+} influx, since La^{3+} ions completely block Ca^{2+} uptake (Borbolla & Peña, 1980). (b) Uptake was measured for short time-intervals (2–20 s) at 25 °C, and was found to be linear with time ($r = 0.91$). Extrapolation of the line to zero-time yielded a similar value to that obtained by 'zero-time' determination (792 and 815 c.p.m. respectively). When transport was measured at 30 °C uptake was linear with time up to 15 s, but the line extrapolated to a similar value (804 c.p.m.). (c) Ca^{2+} uptake was measured in the absence of glucose and in the presence of metabolic

Table 1. *Distribution of $^{45}\text{Ca}^{2+}$ between cytosol, vacuoles and bound $^{45}\text{Ca}^{2+}$ after influx for different times*

Each measurement was repeated three times. Similar results were obtained in three different experiments.

Duration of $^{45}\text{Ca}^{2+}$ influx	Cytosolic free $^{45}\text{Ca}^{2+}$		Vacuolar free $^{45}\text{Ca}^{2+}$		Bound $^{45}\text{Ca}^{2+}$		Total cellular $^{45}\text{Ca}^{2+}$ (c.p.m. per 10^8 cells)
	c.p.m. per 10^8 cells	Percent- age of total	c.p.m. per 10^8 cells	Percent- age of total	c.p.m. per 10^8 cells	Percent- age of total	
'Zero-time'	14	0.9	—	—	1524	99.1	1538
20 s	1985	56.1	2	0.05	1549	43.8	3536
3 min	334	3.7	6179	68.5	2498	27.7	9011

inhibitors. The values obtained were similar to those obtained by 'zero-time' measurements (Fig. 2). It was concluded that 'zero-time' measurements represented $^{45}\text{Ca}^{2+}$ binding and can be subtracted from $^{45}\text{Ca}^{2+}$ transport data to obtain values for Ca^{2+} influx across cell membranes.

To determine whether the first component of Ca^{2+} influx represented Ca^{2+} transport across the plasma membrane and the second represented uptake into the vacuole, cells were preincubated in buffer-glucose to obtain non-steady-state conditions; $^{45}\text{Ca}^{2+}$ uptake was then measured for 0, 20 or 180 s and terminated by addition of La^{3+} . The cells were then immediately cooled to 0°C and subjected to differential extraction. (This was done as fast as possible to minimize subsequent transport of Ca^{2+} from the cytoplasmic pool into the vacuole.) It was found that the amount of $^{45}\text{Ca}^{2+}$ associated with the cells after 'zero-time' measurement was equal to the amount of bound $^{45}\text{Ca}^{2+}$ determined by methanol extraction after 20 s of uptake, and somewhat less than that determined after 3 min. No $^{45}\text{Ca}^{2+}$ in the cytoplasmic or vacuolar pools was observed after the 'zero-time' procedure. After 20 s $^{45}\text{Ca}^{2+}$ was distributed between the cytoplasmic pool (56.1%) and bound $^{45}\text{Ca}^{2+}$ (43.8%). The vacuolar pool did not contain any $^{45}\text{Ca}^{2+}$ (less than 0.1%). After 3 min of the uptake the total amount of $^{45}\text{Ca}^{2+}$ associated with the cells increased by 2.5-fold as compared with the value obtained after 20 s (see also Fig. 1). The amount of bound $^{45}\text{Ca}^{2+}$ increased, probably due to binding to intravacuolar sites (27.7% of total); most of the $^{45}\text{Ca}^{2+}$ was localized in the vacuolar pool (68.5%) and only 3.7% of the cellular $^{45}\text{Ca}^{2+}$ was localized in the cytoplasmic pool (Table 1). It was concluded that under non-steady-state conditions, values measured during the first 20 s of uptake (after subtracting the 'zero-time' measurements) represented transport across the plasma membrane. Measurements of Ca^{2+} uptake after 3 min of uptake represent mainly accumulation into the vacuole. The uptake into the vacuole was inhibited by the addition of ZnCl_2 (0.1 mM) (Fig. 2) which greatly reduced the slope of the second component of Ca^{2+} influx. Inhibition of the vacuolar Ca^{2+} transport system by Zn^{2+} was reported by Ohsumi & Anraku (1983).

Analysis of the non-steady-state kinetics

The time-courses obtained under non-steady-state conditions were analysed in terms of two compartments for Ca^{2+} according to equation (1)

$$\frac{dC}{dt} = A \exp(-\lambda_1 t) + B \exp(-\lambda_2 t) \quad (1)$$

where C is the amount of $^{45}\text{Ca}^{2+}$ taken up by the cells, A and B are the intercepts and λ_1 and λ_2 are the exponential constants of the double exponential equation describing Ca^{2+} uptake by the cells. Thus

$$C = \frac{A}{\lambda_1} [1 - \exp(-\lambda_1 t)] + \frac{B}{\lambda_2} [1 - \exp(-\lambda_2 t)] \quad (2)$$

The curves simulated by computer using equation (2) were similar to the experimental results (Fig. 1) only if we let B increase with time according to the empirical function

$$B = B' \frac{\left(\frac{t}{300}\right)^3}{\left(\frac{t}{300}\right)^3 + 1} \quad (3)$$

where B' is a constant value. This empirical equation expresses a situation where the uptake into the second compartment increases gradually after a short lag-time. Thus using equations (2) and (3) we obtain

$$C = \frac{A}{\lambda_1} [1 - \exp(-\lambda_1 t)] + \frac{B' \cdot \left(\frac{t}{300}\right)^3}{\lambda_2 \cdot \left(\frac{t}{300}\right)^3 + \lambda_2} [1 - \exp(-\lambda_2 t)] \quad (4)$$

Fig. 3 shows a curve simulated by computer using equation (4) and the following values: $\lambda_1 = 0.075$; $\lambda_2 = 0.0075$; $A = 0.15$ and $B' = 0.075$. The simulated curve is composed of two components similar to the experimental curve (Fig. 1). During the first phase (50 s) uptake is only into the first compartment and is represented by

$$C = \frac{A}{\lambda_1} [1 - \exp(-\lambda_1 t)]$$

The second phase mainly represents uptake into the second compartment since uptake into the first compartment reached a constant value (Fig. 3). These simulated kinetics are similar to the results given in Table 1, which show that during the first 20 s Ca²⁺ uptake was only into the cytoplasmic pool, whereas after 3 min uptake was mainly into the vacuolar pool. It does not show the decrease in C in the first compartment between 20 s and 3 min (Table 1).

The non-steady-state transport measurements allowed examination of the mechanism of transport across the plasma membrane in intact cells. We investigated the effect of $\Delta\psi$ on this transport.

⁴⁵Ca²⁺ uptake driven by $\Delta\psi$

At low concentrations TFP causes hyperpolarization of the yeast plasma membrane (Eilam, 1984) by activation of an energy-dependent pump which extrudes K⁺ from the cells (Eilam *et al.*, 1985c). Here we used TFP to produce different $\Delta\psi$ values by preincubating cells with TFP and subsequently washing with buffer. Under these conditions the cells had a high $\Delta\psi$ which was gradually dissipated during 60 min incubation.

In order to examine whether TFP had been removed from the cells by washing, we measured the fluorescence associated with the cells. TFP has a fluorescence peak at 500 nm when excited at 306 nm, but there was no fluorescence associated with the cells after 30 min incubation in 40 μ M-TFP solution and subsequent washing in buffer and resuspension in buffer without TFP. This indicated that the washing procedure removed all TFP from the cells: therefore, the measured effects could be attributed to the high $\Delta\psi$ and not to TFP which may have remained bound to the cells.

Preincubation with TFP caused substantial ⁴⁵Ca²⁺ uptake by cells depleted of ATP, whereas when cells were not preincubated with TFP, Ca²⁺ uptake was almost zero (Fig. 4). Ca²⁺ uptake in the presence of glucose was lower than that driven by the imposed $\Delta\psi$. When metabolic inhibitors were omitted and glucose was added during Ca²⁺ uptake into cells preincubated with TFP, the rate of Ca²⁺ influx was not affected. La³⁺ completely inhibited Ca²⁺ uptake driven by the imposed $\Delta\psi$ or by glucose.

Different values of $\Delta\psi$ were produced as described in Methods. Two types of measurements were made. (1) The dependence of the initial rate of Ca²⁺ uptake (V) on $\Delta\psi$. This was

Fig. 3

Fig. 4

Fig. 3. Computer simulation of the time-course of Ca^{2+} uptake. The simulations were based on equation (4) (see Results) using values of $A = 0.15$, $B' = 0.075$, $\lambda_1 = 0.075$ and $\lambda_2 = 0.0075$. (a) Simulation according to equation (4); (b) each of the components of equation 4 presented separately.

Fig. 4. Time-course of $^{45}\text{Ca}^{2+}$ influx into yeast cells with a high $\Delta\psi$. Cells were preincubated in buffer-glucose medium to obtain non-steady-state conditions followed by preincubation with TFP ($35\text{ }\mu\text{M}$) where indicated to obtain cells with a high $\Delta\psi$. $^{45}\text{Ca}^{2+}$ influx was measured, as described in Methods, at $30\text{ }^\circ\text{C}$ in media containing MES/Tris (10 mM, pH 6.0) and CaCl_2 (10^{-6} M) labelled with $^{45}\text{Ca}^{2+}$ [$0.5\text{ }\mu\text{Ci ml}^{-1}$ (18.5 kBq ml^{-1})]. $\blacktriangle$, Cells preincubated (30 min, $30\text{ }^\circ\text{C}$) with 100 mM-glucose and $35\text{ }\mu\text{M}$ -TFP; transport measured in the presence of glucose. $\triangle$, Cells preincubated with glucose and TFP; transport measured in the absence of glucose and the presence of $15\text{ }\mu\text{M}$ -antimycin A and 5 mM-2-deoxyglucose. $\bullet$, Cells preincubated with glucose only; transport measured in the presence of glucose. $\circ$, Cells preincubated with glucose only; transport measured in the absence of glucose and the presence of $15\text{ }\mu\text{M}$ -antimycin A and 5 mM-2-deoxyglucose. $\blacksquare$, Cells preincubated with glucose and TFP; transport measured in the presence of glucose and 0.1 mM - La^{3+} . $\square$, Transport measured at $0\text{ }^\circ\text{C}$ in the absence of glucose.

determined by measuring Ca^{2+} uptake for 20 s and subtracting the values for Ca^{2+} binding. (2) The dependence of steady-state levels of the first uptake component (L) on $\Delta\psi$. This was determined by measuring uptake for 60 s and subtracting the binding.

The results in Figs 5 and 6 are expressed as $\log(V)$ or $\log(L)$ versus $\Delta\psi$ measured in the same cells. Both $\log(V)$ and $\log(L)$ were linear with respect to $\Delta\psi$ ($r = 0.90$ and 0.95). Similar linear plots were obtained in eight different experiments. The threshold value of $\Delta\psi$ which did not support Ca^{2+} influx in the absence of a metabolic substrate was determined in nine experiments, by extrapolating the linear plots of V against $\Delta\psi$, using $\Delta\psi$ values between -78 and -110 mV . The threshold value obtained was $69.5 \pm 1.2\text{ mV}$.

DISCUSSION

The mechanism of Ca^{2+} influx into yeast cells is not yet understood. Ca^{2+} influx proceeds only in the presence of a metabolic substrate (Boutry *et al.*, 1977) but the driving force for Ca^{2+} influx is not known; ATP, $\Delta\psi$ and $\Delta\bar{\mu}_{\text{H}^+}$ have been suggested (Borst-Pauwels, 1981). In recent studies the kinetic parameters of Ca^{2+} influx and the effects of ions and inhibitors on Ca^{2+} transport and binding have been determined (Roomans *et al.*, 1979; Borbolla & Peña, 1980). The effects of surface potential on the concentration dependence of Ca^{2+} influx have been investigated (Borst-Pauwels & Theuvenet, 1984).

When Ca^{2+} influx is studied in intact cells under steady-state conditions, it is difficult to distinguish between transport across the plasma membrane and across the vacuolar membrane.

Fig. 5

Fig. 6

Fig. 5. Initial rate of $^{45}\text{Ca}^{2+}$ influx as a function of $\Delta\psi$. Cells were preincubated in buffer-glucose medium to obtain non-steady-state conditions followed by preincubation with TFP at concentrations between 0 and $30\ \mu\text{M}$ to obtain cells with different $\Delta\psi$ values. All preincubation media and the transport medium contained $[^3\text{H}]\text{TPP}^+$ [$1\ \mu\text{M}$; $0.05\ \mu\text{Ci ml}^{-1}$ ($1.85\ \text{kBq ml}^{-1}$)]. The cells were washed and transport was measured for 20 s at $25\ ^\circ\text{C}$, as described in Methods, in media containing MES/Tris buffer (10 mM, pH 6.0), antimycin A ($15\ \mu\text{M}$) and 2-deoxyglucose (5 mM). V ($10^{-14} \times \text{mol Ca}^{2+}$ per 10^8 cells·s) was calculated from the 20 s and zero-time uptake data. $\Delta\psi$ was calculated from $[^3\text{H}]\text{TPP}$ accumulation in the same cells.

Fig. 6. Steady-state level of the first $^{45}\text{Ca}^{2+}$ uptake component as a function of $\Delta\psi$. Conditions were as in Fig. 5, but the $^{45}\text{Ca}^{2+}$ uptake was measured for 60 s. L ($10^{-12} \times \text{mol Ca}^{2+}$ per 10^8 cells) represents the steady-state level of Ca^{2+} uptake as determined from 60 s and zero-time transport measurements.

Fig. 7. Computer simulation of the effect of an increase in A (equation 4) on the general shape of the time-course of Ca^{2+} influx. The values used for the simulation were as in Fig. 3, except that for curve (b) the value of A was 0.25.

In the present study we measured Ca^{2+} influx in cells which were preincubated in medium without Ca^{2+} and in which influx was initiated by the addition of Ca^{2+} to the medium. Under these non-steady-state conditions the time-course of Ca^{2+} influx was composed of a saturable component which represented uptake into the cytosol and a linear component which was initiated 60–90 s later and represented uptake into the vacuole (Fig. 1, Table 1). To explain this type of kinetics we may assume that during the preincubation in Ca^{2+} -free medium the concentration of cytosolic Ca^{2+} decreased considerably. During uptake, the concentration of cytosolic Ca^{2+} increased and led to an increase in the rate of uptake into the vacuole. The time-course of Ca^{2+} uptake comprising two components indicates that the increase in the rate of

uptake into the vacuole must be sigmoidal; otherwise two components would not be distinguished.

The non-steady-state kinetics enabled us to investigate the mechanism of Ca^{2+} transport across the plasma membrane by examining the initial rate of Ca^{2+} uptake. In the energy-depleted cells there was no Ca^{2+} influx and the $\Delta\psi$ was -72 mV. When we increased the value of $\Delta\psi$ by preincubation with TFP, substantial Ca^{2+} influx was observed. The general shape of the time-course curve (i.e. presence of two components and a plateau at 60–90 s) was similar to the time-course of Ca^{2+} uptake in the presence of metabolic substrate, but both the rate of uptake and the steady-state level of the first component were much higher. Computer simulations using equation (4) indicated that an increase in the rate and the steady-state level of the first component without a change in the general shape of the curve could be obtained when we let A increase with no change in λ_1 (Fig. 7). Since non-steady-state conditions were maintained during influx, kinetic analysis of the meaning of the increase in A was not possible. It appears that A is a function both of the rate of uptake and the size of the cytosolic compartment (Uchikawa & Borle, 1981). When we plotted the log of the Ca^{2+} influx rate or the log of the steady-state level of Ca^{2+} uptake against $\Delta\psi$ we obtained straight lines. The threshold value of $\Delta\psi$ that did not support Ca^{2+} influx in the absence of metabolic energy was -69.5 mV. We therefore suggest the following mechanism. Ca^{2+} influx across the plasma membrane may be mediated by gated channels which open when $\Delta\psi$ is below a threshold value of -69.5 mV (in our cells). Ca^{2+} influx across the plasma membrane is driven by $\Delta\psi$ so that both the rate of uptake and the size of the cytosolic compartment are a function of $\Delta\psi$. Ca^{2+} which are taken into the cytosol are subsequently taken up by the vacuole by coupling to $\Delta\bar{\mu}_{\text{H}^+}$ across the vacuolar membrane.

In energy-depleted cells in which Ca^{2+} influx into the cytoplasmic compartment was driven by $\Delta\psi$ there was a substantial uptake of Ca^{2+} into the vacuole, but what is the source of energy for the translocation of Ca^{2+} across the vacuolar membrane? We suggest that during the time of the experiment $\Delta\bar{\mu}_{\text{H}^+}$ across the vacuolar membrane is maintained. Similarly, we observed previously that Ca^{2+} uptake into the vacuoles of permeabilized cells proceeds in the absence of added ATP or glucose when fresh preparations are used ($\Delta\bar{\mu}_{\text{H}^+}$ still maintained) but not after 2 h preincubation (Eilam *et al.*, 1985a).

It is suggested from the present results that the plasma membrane functions as a barrier for Ca^{2+} influx or exchange under conditions where there is no metabolic energy, via a mechanism which involves the closure of Ca^{2+} channels at low $\Delta\psi$. The function of the plasma membrane as a barrier to Ca^{2+} uptake was also suggested in our previous work using substances which increased the permeability of the plasma membrane to Ca^{2+} (Eilam *et al.*, 1985d). The Ca^{2+} channel in the plasma membrane may be opened by the increase in $\Delta\psi$ or by addition of metabolic substrates. We do not yet know if addition of glucose causes the opening of Ca^{2+} channels by an increase in $\Delta\psi$ above the threshold value, or via a different mechanism. This problem is currently being investigated in our laboratory.

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