

# Inositol 1,4,5-trisphosphate releases $\text{Ca}^{2+}$ from vacuolar membrane vesicles of *Saccharomyces cerevisiae*

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Inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) induces a release of  $\text{Ca}^{2+}$  from vacuolar membrane vesicles of *Saccharomyces cerevisiae*. The amount released is dependent on  $\text{IP}_3$  concentration (concentration for half maximal effect,  $K_{m, \text{apparent}} = 0.4 \mu\text{M}$ ). *Myo*-inositol, and inositol 1,4-bisphosphate up to  $50 \mu\text{M}$  have no effect on  $\text{Ca}^{2+}$  levels in the vesicles. The  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release is blocked by dantrolene and 8-(*N,N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate-HCl (TMB-8), which are known to block  $\text{Ca}^{2+}$  release from  $\text{Ca}^{2+}$  stores in animal cells.  $\text{IP}_3$ -induced release of  $\text{Ca}^{2+}$  also occurs when  $\text{Ca}^{2+}$  is accumulated by means of an artificial pH gradient, indicating that the effect of  $\text{IP}_3$  is not due to an effect on the vacuolar  $\text{H}^+$ -ATPase. The  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release is not accompanied by a change in the pH gradient, which indicates that it is not due to a reversal of the  $\text{Ca}^{2+}/\text{nH}^+$  antiport or to a decrease in  $\Delta\text{pH}$  by  $\text{IP}_3$ . The present results suggest that  $\text{IP}_3$  may act as a second messenger in the mobilization of  $\text{Ca}^{2+}$  in yeast cells. As in plant cells, the vacuolar membrane of yeast seems to contain a  $\text{Ca}^{2+}$  channel, which can be opened by  $\text{IP}_3$ . In this respect the vacuole could function as an  $\text{IP}_3$ -regulated intracellular  $\text{Ca}^{2+}$  store, equivalent to the endoplasmic- and sarcoplasmic reticulum in animal cells, and play a role in  $\text{Ca}^{2+}$ -dependent signal transduction in yeast cells.

Inositol 1,4,5-trisphosphate-induced  $\text{Ca}^{2+}$  release; Vacuole; *Saccharomyces cerevisiae*

## 1. INTRODUCTION

The free cytosolic  $\text{Ca}^{2+}$  concentration is a major regulatory element in eukaryotic cells [1]. Functioning of  $\text{Ca}^{2+}$  in signal transduction pathways is dependent upon maintaining a low free cytosolic  $\text{Ca}^{2+}$  concentration, while at the same time permitting a rapid and transient increase in this concentration [2,3].

In yeast cells the role of  $\text{Ca}^{2+}$  in signal transduction has only begun to be elucidated. Cytosolic  $\text{Ca}^{2+}$  is found to play an essential role in the control of the cell cycle, in the budding process, and in the late reaction to the mating pheromone  $\alpha$  [4–6]. Furthermore all the elements required for the functioning of a  $\text{Ca}^{2+}$  signal transduction pathway are present in yeast. Calmodulin, adenylate-cyclase, GTP-regulatory proteins and protein kinases in yeast are found to have similar functions as their mammalian counterparts [7–9]. Furthermore, like in mammalian cells, cytosolic free  $\text{Ca}^{2+}$  concentrations in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and the mycelial fungus *Neurospora crassa* are maintained at  $0.1$ – $0.3 \mu\text{M}$  [10–12].

Maintenance of a low cytosolic  $\text{Ca}^{2+}$  concentration in most eukaryotic cells is achieved by  $\text{Ca}^{2+}$  ATPases in addition to  $\text{Na}^+/\text{Ca}^{2+}$  exchangers [13–15] in the plasma membrane, and uptake into intracellular organelles like mitochondria and endoplasmic (ER) and sarcoplasmic

reticulum (SR) in animal cells [2,3,16] and vacuoles in plant cells [17]. In fungi, however,  $\text{Ca}^{2+}$  extrusion by the cells has been ascribed to be mediated by a  $\text{Ca}^{2+}/\text{nH}^+$  exchanger [18,19]. The recent identification of a yeast gene whose product shows strong homology to plasma membrane  $\text{Ca}^{2+}$ -ATPases of animal cells [20], indicates that  $\text{Ca}^{2+}$  extrusion by yeast cells is mediated by a  $\text{Ca}^{2+}$ -ATPase that exchanges  $\text{Ca}^{2+}$  for protons [12].

In yeast cells, like in plant cells, the vacuole is a major calcium store with a free calcium concentration of  $1.3 \text{ mM}$  in *S. cerevisiae* [10]. It has been suggested that in fungi vacuoles are involved in  $\text{Ca}^{2+}$  homeostasis [12,21]. Ohsumi and Anraku showed that  $\text{Ca}^{2+}$  transport into the vacuole is driven by a pH gradient which is generated by the vacuolar  $\text{H}^+$ ATPase [22]. It was found recently, however that disruption in *S. pombe* of *cta3*, a gene whose product shows similarity to the SR  $\text{Ca}^{2+}$ -ATPase in animal cells, affects  $\text{Ca}^{2+}$  homeostasis and transport, leading to the suggestion that other organelles besides the vacuole play a role in  $\text{Ca}^{2+}$  homeostasis as well [11]. As a matter of fact, recently non-vacuolar ATP-dependent intracellular  $\text{Ca}^{2+}$  transport mediated by a calcium pump encoded by the gene *cta3* has been described in the yeast *S. pombe* [23].

It is not known by which mechanisms the mobilization of intracellular  $\text{Ca}^{2+}$  from intracellular stores to the cytoplasm in yeast is achieved. In animal cells, inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ), which is formed by hydrolysis of the membrane lipid phosphatidylinositol 4,5-bisphosphate, can release  $\text{Ca}^{2+}$  from intracellular stores

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[16]. In these cells calcium is released mainly from the ER [16], but in plants also from the vacuole [24]. For fungi there is only one publication that reported on the ability of  $IP_3$  to release  $Ca^{2+}$  from vacuoles in *N. crassa* [25]. Our present findings show that also in yeast  $IP_3$  may cause mobilization of  $Ca^{2+}$  from intracellular stores, notably the vacuole.

## 2. MATERIALS AND METHODS

### 2.1. Strain and culture conditions

The diploid strain A294 (Whitbread & Co., UK) was used throughout. Cells were grown in YEPD medium (1% yeast extract, 2% bacto-peptone and 2% glucose) in 250 ml batch cultures at 30°C on an orbital shaker at 120 r.p.m.

### 2.2. Preparation of vacuolar membrane vesicles

Preparation of vacuolar membrane vesicles was essentially according to Ohsumi and Anraku [26]. First vacuoles were isolated, which were almost free of any contamination as checked by phase contrast microscopy. The vacuoles were converted to right-side-out vesicles through mixing with a buffer of low osmotic strength, buffer A (10 mM MES-Tris pH 6.9, 5 mM  $MgCl_2$  and 25 mM KCl). The vesicles were recovered by centrifugation and stored at -80°C. Purity of the prepared vesicles was checked by measuring the amount of ATPase activity which could be inhibited by vanadate (10  $\mu M$ ), which inhibits the plasma membrane  $H^+$ -ATPase, or azide (2 mM), which inhibits the mitochondrial ATPase [27].

### 2.3. Generation and monitoring of pH gradient

For an ATP-dependent pH gradient vesicles (5–10  $\mu g$  protein/ml, final concentration) were diluted in buffer A containing 98 ng/ml of the pH-sensitive fluorescent dye 9-amino-6-chloro-2-methoxyacridine (ACMA). The reaction was started by the addition of 1.7 mM ATP. The formation of the pH gradient was quantitatively assayed by quenching of ACMA fluorescence according to Okorokov et al. [28], in a quartz cuvet at 20°C, using an Aminco SPF 500 spectrofluorimeter at excitation and emission wavelengths of 410 and 482 nm, respec-

tively. 60 nM valinomycin, 0.12  $\mu M$  nigericin and 1.7  $\mu g/ml$  CCCP (final concentrations) were added from stock solutions in ethanol, with a final concentration of less than 0.2% (v/v) ethanol.

A pH gradient could be generated artificially in the absence of ATP through the formation of a  $K^+$  gradient, by diluting the vesicles containing 25 mM KCl in a  $K^+$ -free buffer, which was converted to a pH gradient using the  $K^+/H^+$  ionophore nigericin [29]. The vesicles were preincubated during 30 s with 0.12  $\mu M$  nigericin (final concentration). The formation of the artificially generated pH gradient was started by diluting vesicles (10  $\mu g$  protein/ml in buffer A) 300-fold in buffer B (10 mM MES-Tris pH 6.9 and 40 mM choline chloride) containing 98 ng ACMA/ml.

Divalent cations were added as dichloride salts from 1 or 0.1 M stock solutions.

### 2.4. $Ca^{2+}$ loading of the vesicles

$Ca^{2+}$  loading with an ATP-dependent pH gradient was performed in buffer A at 20°C. The vesicles (5–20  $\mu g$  protein/ml) were preincubated with 2.2 mM ATP during 100 s, and the reaction was started by the addition of  $^{45}CaCl_2$  (approximately 56 kBq/ml) together with 5  $\mu M$   $CaCl_2$  (final concentration). Samples (100  $\mu l$ ) were diluted in 5 ml ice-cold stopping buffer (10 mM MES-Tris, pH 6.9, 40 mM choline-chloride and 250  $\mu M$   $LaCl_3$ ) and the mix was filtered through Millipore filters (0.45  $\mu m$  pore size). Radioactivity was determined by liquid scintillation counting.

For  $Ca^{2+}$  loading with an artificial pH gradient, vesicles (10–30  $\mu g$  protein/ml) were diluted 100-fold in buffer B containing 0.3  $\mu M$  nigericin and preincubated for 100 s. Further the assay was performed as above.

### 2.5. Chemicals

$^{45}CaCl_2$  was obtained from Amersham International (Amersham, UK). Yeast extract and Bacto-peptone were from Difco (UK). Inositol 1,4,5-trisphosphate, inositol 1,4-bisphosphate, *myo*-inositol and  $Na_2ATP$  (vanadate-free) were from Sigma (USA). Zymolyase was from Seikagaku Kogyo Co (Japan). ACMA was a gift from Prof. Dr. R. Kraayenhof at the Vrije Universiteit Amsterdam. All other chemicals were of analytical grade and obtained from commercial sources.

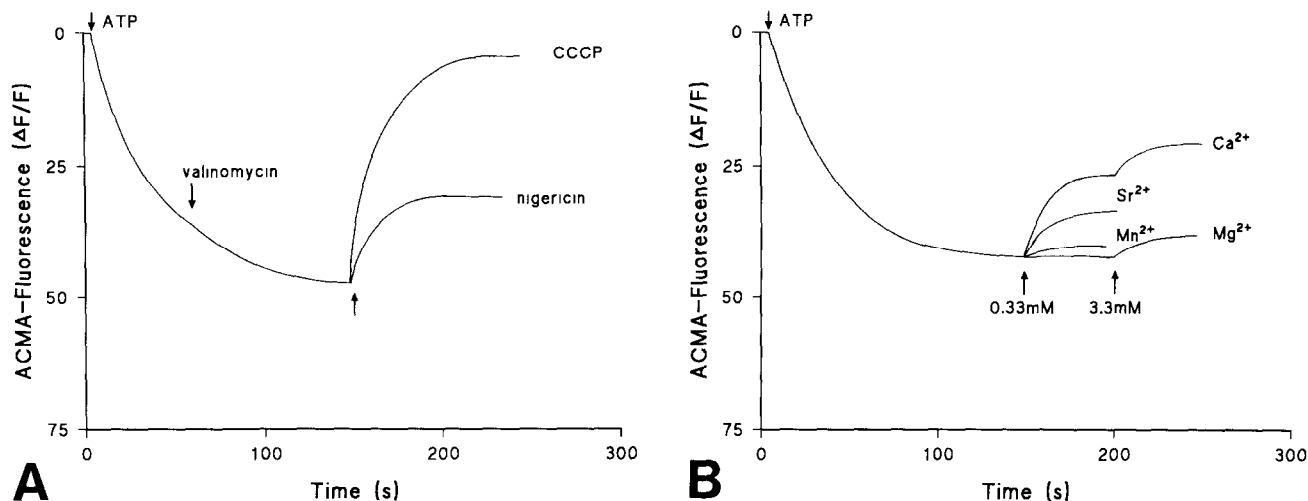


Fig. 1. Proton uptake driven by ATP and dissipation of the resulting pH gradient by nigericin, CCCP and divalent cations. Formation of a  $\Delta pH$  (acid inside) was followed by the quenching of ACMA fluorescence as described in section 2. Incubations were started by the addition of 1.7 mM ATP to the vesicles in buffer A also containing ACMA. (A) 60 nM valinomycin followed by either, 0.12  $\mu M$  nigericin or 1.7  $\mu g/ml$  CCCP were added at the times indicated by the arrows. (B) Dissipation of the  $\Delta pH$  by addition at the time indicated by the first arrow of 0.33 mM  $MgCl_2$ ,  $MnCl_2$ ,  $SrCl_2$  or  $CaCl_2$  followed in the case of  $MgCl_2$  and  $CaCl_2$  by a further dissipation through the addition at the time indicated by the second arrow of 3.3 mM  $MgCl_2$  or  $CaCl_2$ . The tracings are representative of three experiments done in duplicate.

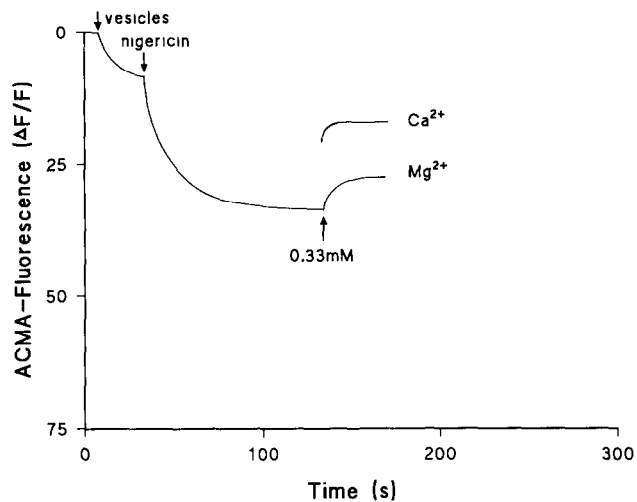


Fig. 2. Proton uptake driven by  $K^+$  gradient and nigericin and dissipation of the resulting pH gradient by  $Ca^{2+}$  and  $Mg^{2+}$ . Formation of  $\Delta pH$  (acid inside) was followed by measurement of the quenching of ACMA fluorescence as described in section 2. Incubation was started by diluting the vesicles 300-fold in buffer B also containing ACMA. Nigericin and  $CaCl_2$  or  $MgCl_2$  were added at the times indicated by the arrows. See also the legend to Fig. 1.

### 3. RESULTS

#### 3.1. ATP-dependent $\Delta pH$ formation

As the vacuolar  $H^+$ -ATPase is not inhibited by vanadate or azide [27], these inhibitors of plasma membrane and mitochondrial ATPase activities, respectively [30], were used to determine the purity of our vacuolar membrane preparation. Inhibition of the ATPase activity of the vacuolar vesicles by vanadate and azide combined was less than 8%, showing that contamination by plasma membranes or mitochondria was low.

In the presence of ATP and  $Mg^{2+}$  a stable  $\Delta pH$  (interior acid) was formed as measured by the quenching of ACMA fluorescence. Valinomycin gave a small increase in the  $\Delta pH$  formed, while protonophore CCCP totally and  $H^+/K^+$  exchanger nigericine partly dissipated the  $\Delta pH$  (Fig. 1A). The  $\Delta pH$  was partly dissipated by  $Ca^{2+}$  (depending upon the  $Ca^{2+}$  concentration added) until a new stable  $\Delta pH$  plateau was reached.  $Sr^{2+}$  had a similar effect upon  $\Delta pH$  though was less effective than  $Ca^{2+}$ .  $Mn^{2+}$  was still less effective in dissipating the pH gradient, while  $Mg^{2+}$  only slightly decreased  $\Delta pH$  (Fig. 1B).  $Cd^{2+}$  also dissipated the  $\Delta pH$  effectively, but after the initial fast decrease the pH gradient kept slowly dissipating (results not shown).

#### 3.2. Artificially generated $\Delta pH$ formation

We also used an artificially generated pH gradient (interior acid) by loading vesicles with KCl, diluting in buffer without  $K^+$  and  $Mg^{2+}$  and using nigericin to convert the  $K^+$  gradient to a proton gradient. Already the addition of the vesicles to the buffer caused a small

quenching of the ACMA fluorescence, indicating that a small pH gradient (interior acid) was formed. This quenching was reduced by addition of  $Mg^{2+}$  and was not found if the vesicles were added to buffer without  $K^+$  but with 5 mM  $Mg^{2+}$ . Possibly the small  $\Delta pH$  was formed as a consequence of diluting the  $Mg^{2+}$  containing vesicle suspension (5 mM  $MgCl_2$ ) in  $Mg^{2+}$ -free buffer. After addition of nigericin a further  $\Delta pH$  formation took place. The  $\Delta pH$  formed was smaller than with ATP and also less stable (it was stable for about 10–20 min, whereas the ATP-generated pH gradient was stable for over an hour).  $Ca^{2+}$  partly dissipated the  $\Delta pH$  as with the ATP-dependent  $\Delta pH$ , but the plateau was reached very fast (Fig. 2).  $Mg^{2+}$  also dissipated the  $\Delta pH$  though less effective and not as fast as  $Ca^{2+}$ .

#### 3.3. $IP_3$ stimulated $Ca^{2+}$ release in Mg-ATP energized conditions

Accumulation of  $^{45}Ca^{2+}$  by an ATP-dependent pH gradient is shown in Fig. 3. It proceeded only in the presence of  $Mg^{2+}$  and ATP. The  $^{45}Ca^{2+}$  accumulated was released completely after addition of 0.01% (w/v) Triton X-100 (not shown) or 0.5  $\mu g/ml$  A23187, a  $Ca^{2+}$ -ionophore (Fig. 3), showing that the  $Ca^{2+}$  which was accumulated was not bound irreversibly inside the vesicles.

Addition of  $IP_3$  to vesicles which had accumulated  $Ca^{2+}$  driven by the ATP-dependent  $\Delta pH$ , resulted in a transient loss of up to 40% of the  $Ca^{2+}$  accumulated in the vesicles (Fig. 3). The maximum amount of  $Ca^{2+}$  release was reached within 45–60 s after addition of  $IP_3$ , thereafter part of the  $Ca^{2+}$  lost was taken up again. After about 200 s the  $Ca^{2+}$  concentration was recovered again partly. A second addition of  $IP_3$  at this point

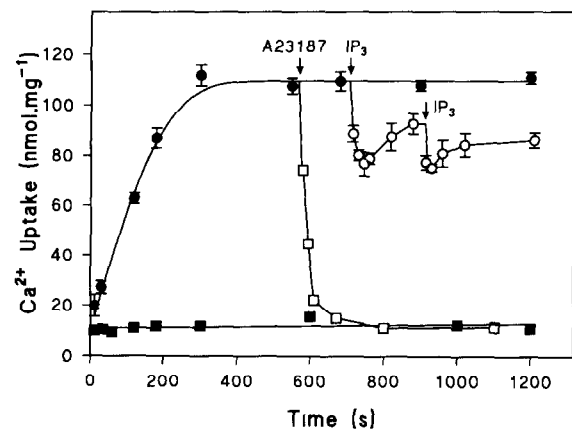


Fig. 3. Time course of  $Ca^{2+}$  uptake driven by an ATP-generated pH gradient and effect of  $IP_3$  upon  $Ca^{2+}$  transport in tonoplast vesicles.  $Ca^{2+}$  uptake was assayed in the presence of 2.2 mM ATP and 5  $\mu M$   $Ca^{2+}$  in buffer A. Vesicles were preincubated 100 s with ATP before addition of  $Ca^{2+}$  (●). Control without ATP added (■). At the times indicated by the arrows  $IP_3$  (○, 1  $\mu M$ ) or A23187 (□, 0.5  $\mu g \cdot ml^{-1}$ ) were added. The assay was performed as described in section 2. Means  $\pm$  S.E.M. of triplicates are shown. Error bars were not drawn when smaller than the symbols.

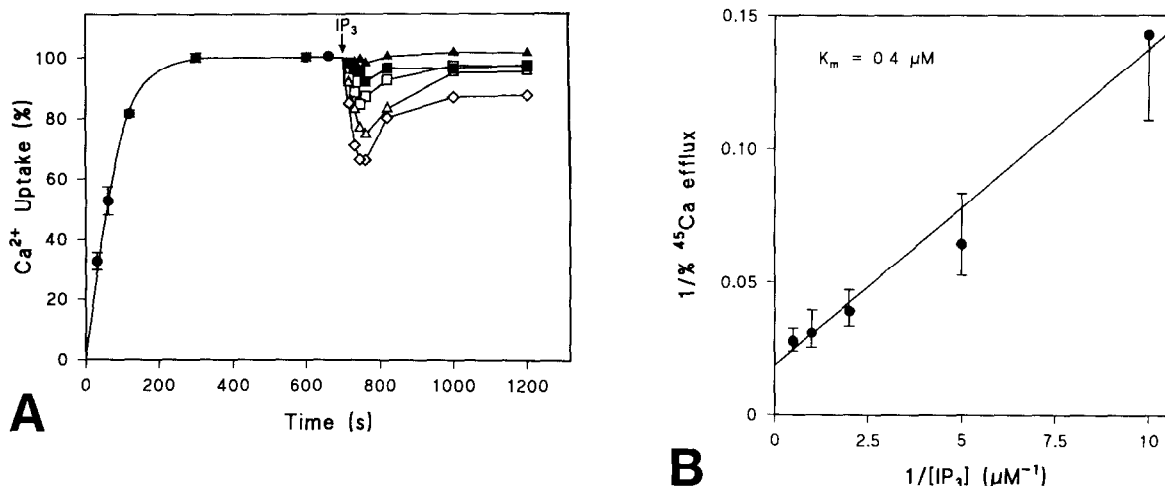


Fig. 4 Effect of IP<sub>3</sub>, TMB-8 and dantrolene on Ca<sup>2+</sup> release from tonoplast vesicles. (A) Ca<sup>2+</sup> (5 μM) was accumulated into the vesicles driven by an ATP dependent  $\Delta$ pH (●), and after equilibrium is reached IP<sub>3</sub> is added at the time indicated by the arrow. Final IP<sub>3</sub> concentration added was 0.1 μM (□), 0.5 μM (▲) or 2 μM (◇). 200 μM TMB8 or 20 μM dantrolene were added 100 s before the addition of 2 μM IP<sub>3</sub>, (■) and (▲), respectively. The assay was performed as described in section 2. (B) The maximum amount of Ca<sup>2+</sup> released (at 45–60 s) by different IP<sub>3</sub> concentrations is plotted in a Lineweaver–Burk plot. Means  $\pm$  S.E.M. of 5 experiments are shown. The concentration of IP<sub>3</sub> needed for half maximal release (0.4 μM) is indicated by the (apparent) K<sub>m</sub>.

again released Ca<sup>2+</sup> followed by re-uptake of the Ca<sup>2+</sup> (Fig. 3). The maximum amount of Ca<sup>2+</sup> released was dependent upon the concentration of IP<sub>3</sub> used (Fig. 4A). Plotting the maximum amount of Ca<sup>2+</sup> released against the IP<sub>3</sub> concentration in a Lineweaver–Burk plot gave a concentration for half-maximum effect of 0.4 μM IP<sub>3</sub> (Fig. 4B).

Specificity of the IP<sub>3</sub> induced Ca<sup>2+</sup> release was tested with two other inositides; *myo*-inositol and inositol 1,4-bisphosphate. Both inositides released less than 5% of the Ca<sup>2+</sup> accumulated up to a concentration of 50 μM, whereas 2 μM IP<sub>3</sub> was enough for near-maximum Ca<sup>2+</sup> release (not shown). Also the IP<sub>3</sub> induced Ca<sup>2+</sup> release could be inhibited for 90% with 20 μM dantrolene (Fig. 4A) which is known to inhibit IP<sub>3</sub> induced Ca<sup>2+</sup> release from SR in animal cells [31,32]. Another inhibitor of IP<sub>3</sub> induced Ca<sup>2+</sup> release is TMB-8 [33,34]. 200 μM TMB-8 inhibited Ca<sup>2+</sup> release from these vesicles for more than 90% (Fig. 4A). TMB-8 nor dantrolene had an effect upon the equilibrium level of Ca<sup>2+</sup> accumulation before addition of IP<sub>3</sub> (not shown).

### 3.4. IP<sub>3</sub> stimulated Ca<sup>2+</sup> release in artificially energized conditions

The level of Ca<sup>2+</sup> accumulation found with the artificial pH gradient was consistently lower than with the ATP-dependent pH gradient, being about 40 nmol·mg<sup>-1</sup> and about 110 nmol·mg<sup>-1</sup>, respectively (see Figs. 3 and 5). This may reflect the difference in the magnitude of the  $\Delta$ pH formed, the artificially formed gradient being the smaller (see Figs. 1 and 2). The accumulation of <sup>45</sup>Ca<sup>2+</sup> into the vacuolar vesicles driven by an artificial pH gradient is shown in Fig. 5. It only proceeded when

nigericin was added. Again A23187 released all the Ca<sup>2+</sup> which was accumulated in the vesicles (Fig. 5). Here IP<sub>3</sub> caused release of about 80% of the Ca<sup>2+</sup> accumulated, and there was no re-uptake. The small amount of Ca<sup>2+</sup> still present in the vesicles could be released by A23187.

Addition of IP<sub>3</sub> to Ca<sup>2+</sup> loaded vesicles driven by both ATP-generated or K<sup>+</sup>/nigericin generated pH gradients did not affect these pH gradients as deduced from ACMA fluorescence during the time measured (20–30 s) (not shown).

## 4. DISCUSSION

Ohsumi and Anraku provided indirect evidence that Ca<sup>2+</sup> can be accumulated into vacuoles by means of the proton gradient generated by the vacuolar H<sup>+</sup>-ATPase [22]. Okorokov et al. [35] showed that vacuolar Ca<sup>2+</sup> uptake proceeds through an electroneutral Ca<sup>2+</sup>/2H<sup>+</sup> antiport. Our work is in accordance with the notion that the pH gradient is the driving force for Ca<sup>2+</sup> uptake. That ATP is only necessary for the generation of the proton gradient and not directly involved in Ca<sup>2+</sup> transport is shown in a more direct way, through the use of an artificially generated pH gradient in the absence of ATP. The pH gradient generated through the use of a K<sup>+</sup> gradient and nigericin is sufficient to drive Ca<sup>2+</sup> uptake into the vacuolar vesicles.

The dissipation of the pH gradient after the addition of Ca<sup>2+</sup> is also found after the addition of Cd<sup>2+</sup>, Sr<sup>2+</sup>, Mn<sup>2+</sup> and Mg<sup>2+</sup>. Addition of Mg<sup>2+</sup> has only a small effect upon the MgATP-generated  $\Delta$ pH but a much larger effect upon the artificially generated  $\Delta$ pH (compare Fig. 1B and Fig. 2). Furthermore, Ca<sup>2+</sup> dissipates

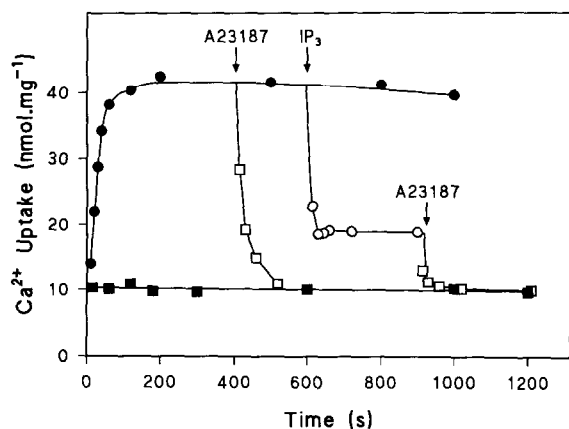


Fig. 5. Time course of  $\text{Ca}^{2+}$  uptake driven by an artificial pH gradient and effect of  $\text{IP}_3$  upon  $\text{Ca}^{2+}$  transport in tonoplast vesicles.  $\text{Ca}^{2+}$  uptake was assayed in buffer B in the presence of  $0.3 \mu\text{M}$  nigericin and  $5 \mu\text{M}$   $\text{Ca}^{2+}$ . Vesicles were preincubated for 100 s with nigericin before addition of  $\text{Ca}^{2+}$  (●). Control without nigericin added (■). At the times indicated by the arrows  $\text{IP}_3$  (○,  $1 \mu\text{M}$ ) or A23187 (□,  $0.5 \mu\text{g}\cdot\text{ml}^{-1}$ ) were added. The assay was performed as described in section 2. The data are an average of 3 experiments.

the pH gradient much faster in case of the artificial  $\Delta\text{pH}$ . These differences are probably due to the presence of  $3.3 \text{ mM}$  free  $\text{Mg}^{2+}$  in the buffer in the case of the ATP-dependent  $\Delta\text{pH}$ , whereas no extra  $\text{Mg}^{2+}$  is added to the buffer applied in case of the artificial  $\Delta\text{pH}$ . The small pH gradient formed after the addition of the vesicles to the buffer in case of the artificial  $\Delta\text{pH}$  (see Fig. 2) can be explained by the movement of  $\text{Mg}^{2+}$  from the vesicles (obtained from a  $5 \text{ mM}$   $\text{Mg}^{2+}$  containing buffer) to the  $\text{Mg}^{2+}$ -free buffer by means of the antiporter. Our results agree with previous findings of Okorokov et al. [35] that other divalent cations, including the physiologically important  $\text{Mg}^{2+}$ , are also transported by the antiporter. Ohsumi and Anraku also used excess  $\text{MgCl}_2$  (about  $3.7 \text{ mM}$  free [22], which may explain why they did not find a decrease in the pH gradient on adding extra  $\text{Mg}^{2+}$ .

The values reported for yeast for the  $K_m$  of the exchanger are in the order of  $60$  to  $100 \mu\text{M}$  [22,35]. These values are well above the value of  $0.1$ – $0.3 \mu\text{M}$  free calcium in the cytosol [10–12]. This led to the suggestion that the vacuolar  $\text{Ca}^{2+}/2\text{H}^+$  exchanger functions as a low-affinity high capacity transporter under conditions of high extracellular  $\text{Ca}^{2+}$  concentrations or in  $\text{Ca}^{2+}$ -detoxification after membrane damage [23,36]. However, in the determination of the affinity of the antiport for  $\text{Ca}^{2+}$ , the fact that  $\text{Mg}^{2+}$  also seems to have affinity for the antiporter, and thus may act as a competitive cation, was not taken into account. Depending on how strong  $\text{Mg}^{2+}$  competes with  $\text{Ca}^{2+}$  for the transport-site and on the free  $\text{Mg}^{2+}$  concentration in the cytosol, the  $K_m$  of the antiport for  $\text{Ca}^{2+}$  in vivo may be much lower than the reported values. If this is the case, the vacuole could still play a physiological role in maintaining a low

cytosolic  $\text{Ca}^{2+}$  concentration. Present investigations are aimed at this problem.

The recent identification of intracellular  $\text{Ca}^{2+}$ -ATPases in yeast [23], led to the suggestion that these  $\text{Ca}^{2+}$ -ATPases are the high affinity  $\text{Ca}^{2+}$ -transporters as they are in animal cells, and that the ER in yeast supplies the  $\text{Ca}^{2+}$  needed for the regulation of cell cycle processes [23]. But as argued above the vacuole could still play a role in high affinity  $\text{Ca}^{2+}$  uptake, possibly in parallel to non-vacuolar compartments in maintaining a low cytosolic  $\text{Ca}^{2+}$  concentration.

In this publication evidence for a possible role of the vacuole as an inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) sensitive  $\text{Ca}^{2+}$  pool is presented. In the presence of ATP,  $\text{IP}_3$  causes a transient release of  $\text{Ca}^{2+}$  from vacuolar vesicles in yeast. The effect of  $\text{IP}_3$  upon the maximum amount of  $\text{Ca}^{2+}$  released is concentration dependent with a  $K_m$  of  $0.4 \mu\text{M}$ . This value is close to the values of  $0.2$ – $0.6 \mu\text{M}$  found for plant vacuoles [24,37,38]. The only other report of an effect of  $\text{IP}_3$  upon  $\text{Ca}^{2+}$  release in fungi describes an increase in  $\text{Ca}^{2+}$  efflux from whole vacuoles of *N. crassa* with a  $K_m$  for  $\text{IP}_3$  of  $5.28 \mu\text{M}$  [25], more than 10 times higher than the value reported here. This discrepancy could be due to a difference in membrane properties or to the different experimental approach.

A possible effect of  $\text{IP}_3$  upon the vacuolar  $\text{H}^+$ -ATPase leading to a transient  $\text{Ca}^{2+}$  release is not very probable because  $\text{IP}_3$  also induces a  $\text{Ca}^{2+}$  release from vesicles where  $\text{Ca}^{2+}$  accumulation is driven by an artificially generated  $\Delta\text{pH}$ . Involvement of the  $\text{Ca}^{2+}/2\text{H}^+$  antiport in the process of  $\text{IP}_3$  induced  $\text{Ca}^{2+}$  release is improbable, because the  $\text{Ca}^{2+}$  release is not accompanied by a  $\Delta\text{pH}$  formation, neither in the vesicles with an ATP-dependent  $\Delta\text{pH}$ , nor in the vesicles with an artificially generated  $\Delta\text{pH}$ . Thus  $\text{IP}_3$  induced  $\text{Ca}^{2+}$  release from yeast vacuoles probably takes place through a separate  $\text{IP}_3$  regulated  $\text{Ca}^{2+}$ -channel.

From the other inositolphosphates tested none caused a significant  $\text{Ca}^{2+}$  release, which is in agreement with the specificity of the  $\text{IP}_3$ -regulated  $\text{Ca}^{2+}$ -channel in animal microsomes and plant vacuoles [16,24,37,38]. The  $\text{IP}_3$  receptor of the putative  $\text{Ca}^{2+}$  channel also shows other physiological similarities with the receptors from animal- and plant-cells in the effects of TMB8 and dantrolene, which both inhibit the  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release. TMB8 and dantrolene are known to inhibit the  $\text{IP}_3$ -activated  $\text{Ca}^{2+}$  release from animal microsomes [31–34], and TMB8 also blocks  $\text{Ca}^{2+}$  release from plant cell vacuoles [24,37,38]. Dantrolene also inhibits the  $\text{IP}_3$  induced  $\text{Ca}^{2+}$  leakage from vacuoles in *N. crassa* [25].

Although several processes in yeast are found to be regulated by the cytosolic  $\text{Ca}^{2+}$  concentration, it is not yet clear what the role of internal  $\text{Ca}^{2+}$  stores in the regulation of this concentration is. In animal cell signaling systems,  $\text{IP}_3$  formed after hydrolysis of membrane phosphoinositide lipids mobilizes  $\text{Ca}^{2+}$  from intracellular stores [16]. In yeast cells the role of hydrolysis and

metabolism of phosphoinositide lipids in signal transduction is not yet clear. Indications for such a role are the enhancement of polyphospho-inositide synthesis by cAMP, which may lead to enhanced production of IP<sub>3</sub> [39], and the fact that the hydrolysis of phosphatidylinositol 4,5-bisphosphate is a requisite process for yeast cell growth [40]. Our results indicate that the vacuolar membrane contains a Ca<sup>2+</sup> channel, which can be opened by IP<sub>3</sub>. This suggests a possible role for IP<sub>3</sub> as a second messenger in mobilizing Ca<sup>2+</sup> from the vacuole in yeast cells.

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