The Effect of Azalomycin F on Ca^{2+} homeostasis in *Trichoderma viride* and *Saccharomyces cerevisiae*

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Abstract. Azalomycin F (AMF), a macrocyclic lactone antibiotic, in concentrations of 10^{-5} g/ml (10^{-6}–10^{-5} mol/l) was found to stimulate both the $^{45}Ca^{2+}$ influx and efflux in intact *Trichoderma viride* submerged mycelium and in cells of *Saccharomyces cerevisiae* without having Ca^{2+} ionophoric properties. AMF also inhibited ATP-dependent Ca^{2+} uptake in membrane fractions prepared from *T. viride* submerged mycelium. $^{45}Ca^{2+}$ which had been accumulated in membrane fractions in an ATP-dependent manner was released upon addition of AMF. This release was observed in light organellar fractions (LOF) of *S. cerevisiae* and of *T. viride* submerged mycelium and, to a small extent, in heavy organellar fraction (HOF) of *S cerevisiae*. No Ca^{2+} releasing effect of AMF was observed in HOF from *T. viride* submerged mycelium. In *S. cerevisiae* expressing Ca^{2+}-dependent photoprotein aequorin, AMF induced transients of luminescence which reflect changes in the cytoplasmic Ca^{2+} concentration. The results suggest that the stimulation by AMF of the Ca^{2+} efflux from the mycelium (cells) could be explained by an increase of the cytoplasmic Ca^{2+} concentration due to the release of Ca^{2+} from microsomal membranes or to the stimulation of Ca^{2+} influx.

Key words: Azalomycin F — Ca^{2+} flux — *Saccharomyces cerevisiae* — *Trichoderma viride*

Abbreviations: A23187 – calcimycin; AMF – azalomycin F; BAPTA – 1,2-bis(2-Aminophenoxy)ethane-N,N,N',N'- tetraacetic acid; $[Ca^{2+}]_{cyt}$ – cytosolic free Ca^{2+} concentration; Cz-D – Czapek-Dox medium; DBP – dibutylphtalate; DOP – dioctylphtalate; drw – dry weight; EDTA – ethylenediaminetetraacetic acid; EGTA – ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid; HOF – heavy (mitochondrial/vacuolar) organellar fraction, LOF – light (microsomal) organellar

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fraction; rlu – relative luminiscence units; SD medium – synthetic dextrose medium; TCA – trichloroacetic acid; TPG – thapsigargin; Tris – tris(hydroxylmethyl) aminomethane; YA – yeast autolysate; YPD – yeast extract-peptone-dextrose medium; wwt – wet weight

Introduction

Azalomycin F (AMF) is a natural compound isolated from *Streptomyces hygroscopicus* (Arai 1960a,b; Chandra and Nair 1995) and *Actinomyces imbricatus* (Tsyganov et al. 1970) and possessing antibiotic properties against fungi (Abou-Gabal and Rieth 1968; Arai 1968), *Trichomonas* (Arai 1968) and bacteria (Sugawara 1968). Indirect evidence has been obtained that this compound interacts with phospholipids (Kuroda et al. 1978). During the screening of numerous compounds for their inhibition of Ca^{2+} influx in *Trichoderma viride* mycelium we found AMF to affect $^{45}Ca^{2+}$ fluxes measured *in vitro*. Here in, we present these observations and show that AMF affects $^{45}Ca^{2+}$ fluxes also in cells and membranes of *Saccharomyces cerevisiae*.

Materials and Methods

Chemicals

The chemicals used were from the following sources: Radionuclide $^{45}CaCl_2$ from Radiochemical Centre (Amersham, U.K.); A23187, thapsigargin and ryanodine from Calbiochem (Luzern, Switzerland); AMF and desertomycin were kindly provided by Professor V. Betina, University of Trnava, Slovakia Chemically synthesized coelenterazine was a generous gift from Professor M. Isobe, Nagoya University, Japan. All other reagents were purchased from Lachema (Brno, Czech Republic).

Strains and culture of cells

Trichoderma viride, strain CCM F-534 from the Czechoslovak Collection of Microorganisms, T. G. Masaryk University, Brno, Czech Republic, was submerged cultured in Czapek-Dox medium with 0.5% (w/v) yeast autolysate (Cz-D+YA) for 20 h at 25 °C and at 4 Hz. *Saccharomyces cerevisiae*, strain W303-1B, was kindly provided by Dr. Gabriela Gavurníková, Department of Biochemistry, Faculty of Natural Sciences, Comenius University, Bratislava and cultured in yeast extract-peptone-dextrose medium (YPD medium) at 30 °C overnight at 4 Hz. For measurement of cytoplasmic free calcium ion concentration ($[Ca^{2+}]_{cyt}$) by aequorin luminescence, strain H208-3B carrying pGAPAQ (Nakajima-Shimada et al. 1991) was grown in a synthetic medium (SD medium) minus tryptophan at 25 °C up to 5×10^6 cells/ml. Human blood samples were purchased from the local blood donor station. Blood was withdrawn on the previous day into acid-citrate-dextrose anticoagulant. Red blood cells were isolated by differential centrifugation into the medium containing: 20 mmol/l Tris-HCl (pH 7.3), 135 mmol/l NaCl, 5 mmol/l KCl

and 10 mmol/l glucose according to the procedure published earlier (Varečka et al. 1997).

Subcellular fractionation

This was done according to the procedure of Cornelius and Nakashima (1987) with several modifications. Twenty-hour old mycelia of *T. viride* were harvested by filtration on a nylon net and washed three times with 0.15 mol/l NaCl containing 25 mmol/l Tris-HCl (pH 7.4). The mycelia (10–20 g wwt) were resuspended in 50 ml of an ice cold homogenization medium containing 0.5 mol/l mannitol, 25 mmol/l Tris-HCl (pH 7.4), 0.1 mmol/l ATP, 1 mmol/l phenylmethylsulfonyl fluoride and 1 mmol/l benzamidine, and disrupted in a homogenizer (MPW 120, Poland) with 15 ml of glass beads for 5 × 1 min. The homogenate was centrifuged at 1,000 × *g* for 20 min to remove cell debris and glass beads, and the resultant supernatant was re-centrifuged at 14,000 × *g* for 30 min. The pellet was used as heavy organellar fraction (HOF) which contained mitochondria and vacuoles, and the supernatant as light organellar fraction (LOF) which contained microsomes. All the above procedures were carried out at 4°C.

The overnight culture of *S. cerevisiae* (30°C, 4 Hz) was centrifuged, and washed with and resuspended in 85 mmol/l sucrose containing 25 mmol/l Tris-HCl (pH 7.4). Three volumes of thick suspension (5 × 10^9 cells/ml) in the above medium were mixed with one volume of glass beads and vortexed for 5 × 30 s with 2 min interruptions to cool the suspension. The homogenate was fractionated as described for *T. viride*.

Following enzymes were used as organelle markers: α-D-mannosidase for the vacuoles (Yoshihisa et al. 1988), GDPase for the Golgi apparatus (Abeijon et al. 1989), NADPH: cytochrome c oxidoreductase for the endoplasmic reticulum (Feldman et al. 1987), and vanadate-sensitive H^+-ATPase for the plasma membrane (Bowman et al. 1978). Cytochrome c oxidase, the marker for mitochondria, was measured spectrophotometrically at 550 nm in 50 mmol/l phosphate buffer (pH 7) containing 0.1% (v/v) Triton X-100. The reaction was started by the addition of the reduced form of cytochrome c.

$^{45}Ca^{2+}$ uptake

Mycelia from submerged cultures were suspended in 85 mmol/l sucrose containing 25 mmol/l Tris-HCl (pH 7.4) to a final concentration of about 4 mg drw/ml. The mycelial suspension showed no apparent heterogeneity. It was kept at room temperature and immediately used for experiments. Aliquots of the suspension were incubated with 0.5 mmol/l $^{45}CaCl_2$ (specific activity about 1,000 cpm/nmol) for 1 h, or the time as indicated in Figures at 25°C. Subsequently, 1 ml aliquots were withdrawn and filtered through a Whatman GF/A glass microfiber filter. The mycelia on the filter were washed with 2 × 4.5 ml of 0.15 mol/l NaCl containing 10 mmol/l EDTA-Tris (pH 7.4). Radioactivity of the mycelia was measured with a liquid scintillation counter (1214 Rackbeta, LKB, Wallac Oy, Sweeden).

The overnight culture of *S. cerevisiae* was washed and resuspended in 85 mmol/l sucrose containing 25 mmol/l Tris-HCl (pH 7.4) at 7×10^8 cells/ml. The suspension was incubated with $^{45}CaCl_2$ as described above and 1 ml aliquots were taken for the liquid scintillation counting. The $^{45}Ca^{2+}$ transport in human red blood cells was measured as described recently (Varečka et al. 1997) after 5 min incubation with both AMF and radionuclide, washing out the external radioactivity with EGTA-containing solution. The radioactivity of trichloroacetic acid (TCA) extracts of red cell pellets has been measured. Hemolysis was determined by the measurement of A_{550} of hemoglobin released from cells by the AMF treatment (5 min, room temperature) after spinning down cells in a microcentrifuge for 1 min.

$^{45}Ca^{2+}$ *efflux*

T. viride was cultured as above in the presence of 1 μCi $^{45}CaCl_2$/ml medium. The 20-h old radiolabelled mycelia were harvested by centrifugation at 5,000 $\times$ *g* for 5 min. The cell pellet was resuspended in Cz-D medium containig 10 mmol/l EDTA without yeast autolysate (Cz-D *minus* YA), and recentrifuged. This step was repeated twice. EDTA remaining in mycelia was washed out three times with Cz-D *minus* YA. The obtained mycelial pellet was suspended in Cz-D *minus* YA to a final concentration of approximately 3 mg dry weight/ml. Only mycelial suspensions without apparent heterogeneity were used for experiments. All experiments were carried out at 25 °C, unless indicated differently.

S. cerevisiae was cultured in the presence of 2 μCi $^{45}CaCl_2$/ml medium. The overnight culture was treated as above except that the cell pellet was washed with 85 mmol/l sucrose containing 25 mmol/l Tris-HCl (pH 7.4) and 10 mmol/l EDTA, and was suspended in the same medium without EDTA at about 5×10^8 cells/ml. All experiments were carried out at 25 °C.

The radiolabelled-mycelia or -cell suspension was incubated at 25 °C under desired conditions. Aliquots (150 μl) of the mycelial suspension were withdrawn, layered on a 2 : 1 (v/v) mixture of dibutylphtalate and dioctylphtalate containing 50 μl of 50 mmol/l EDTA-Tris (pH 7.4) and centrifuged at 5,000 $\times$ *g* for 3 min. The aqueous layer at the top of the mixture was directly taken for liquid scintillation counting. The organic phase was removed and the pellet on the bottom of Eppendorf tube was treated with 10% (w/v) TCA containing 10 mmol/l $LaCl_3$ for 30 min and centrifuged. Radioactivity of the resultant supernatant was counted and expressed as average of triplicates ± standard deviation of a representative of three experiments.

$^{45}Ca^{2+}$ *influx in isolated subcellular fractions*

$^{45}Ca^{2+}$ influx in the isolated subcellular fractions was measured in a reaction mixture containing the membrane fraction (protein concentration, about 1 mg/ml), 0.5 mol/l sorbitol (or mannitol), 25 mmol/l Tris-HCl (pH 7.4), 1 mmol/l Mg^{2+} and 1 mmol/l ATP. The uptake was started by adding $^{45}CaCl_2$ to a final concentration 50 μmol/l (specific activity, *ca.* 10,000 cpm/nmol). At time intervals, 100 μl of the assay mixture were removed and filtered through a nitrocellulose membrane

filter (pore size, 0.6 μm) to collect the subcellular fractions. The filter was then rapidly washed with 2 × 3 ml aliquots of 0.3 mol/l sucrose containing 10 mmol/l EDTA-Tris (pH 7.4) and taken for liquid scintillation counting. The results are expressed as average of duplicates ± standard error of a representative of number of experiments indicated in the legend.

$^{45}Ca^{2+}$ release from subcellular fractions

Membrane fractions were loaded with $^{45}Ca^{2+}$ in the presence of ATP for 15 minutes as described above. After 15 minutes, the tested compounds were added to reaction mixture. At time intervals indicated in Figs. 4 and 5, 100 μl of the assay mixture were removed, filtered through a nitrocellulose membrane filter (pore size 0.6 μm) and washed as above. The radioactivity retained on membrane filters was measured and is shown in Figures. The results are expressed as average of duplicates ± standard error of a representative of number of experiments indicated in the legend.

$[Ca^{2+}]_{cyt}$ measurement by aequorin luminescence

This was done essentially as previously reported (Mori et al. 1998). Briefly, *S. cerevisiae* expressing apoaequorin was harvested and suspended at 1 × 10^8 cells/ml in SD-*minus*-glucose medium to suppress the growth of the cells. To constitute aequorin, the resuspended cells were incubated with 20 μmol/l coelenterazine at 25 °C for 3 h with shaking at 130 rpm. Cells were washed and resuspended in SD-*minus*-glucose medium at 2.5 × 10^7 cells/ml. The coelenterazine-loaded cell suspension (190 μl) was transferred to a plastic tube settled in a luminometer (Lumicounter 1000, Nichion Biotech Co., Funabashi, Japan). Aequorin luminescence induced by AMF was recorded with a pen recorder (Rikadenki Co., Tokyo, Japan), and was expressed as relative luminiscence units (rlu) (Fig. 6).

Protein determination

Proteins were determined by the method of Lowry with bovine serum albumin as standard.

Results

Effect of AMF on Ca^{2+} fluxes in intact cells of T viride mycelium and S cerevisiae

AMF (1–30 μmol/l) stimulated the $^{45}Ca^{2+}$ uptake into the intact submerged mycelium of *T. viride* (Fig. 1A). Higher concentrations of this compound had a lesser stimulatory action showing a maximum stimulation at 10 μmol/l. AMF, at similar concentrations, also stimulated the efflux of $^{45}Ca^{2+}$ from the *T viride* mycelium (Fig. 1B). Similar effects of AMF were observed in *S cerevisiae* In the presence of 40 μmol/l AMF, the $^{45}Ca^{2+}$ uptake dramatically increased (Fig. 1C) The release of $^{45}Ca^{2+}$ from the pre-labelled cells was greatly increased at as high as 20 μmol/l (Fig. 1D). Thus, the effect of AMF on $^{45}Ca^{2+}$ flux is not restricted to the filamentous fungi.

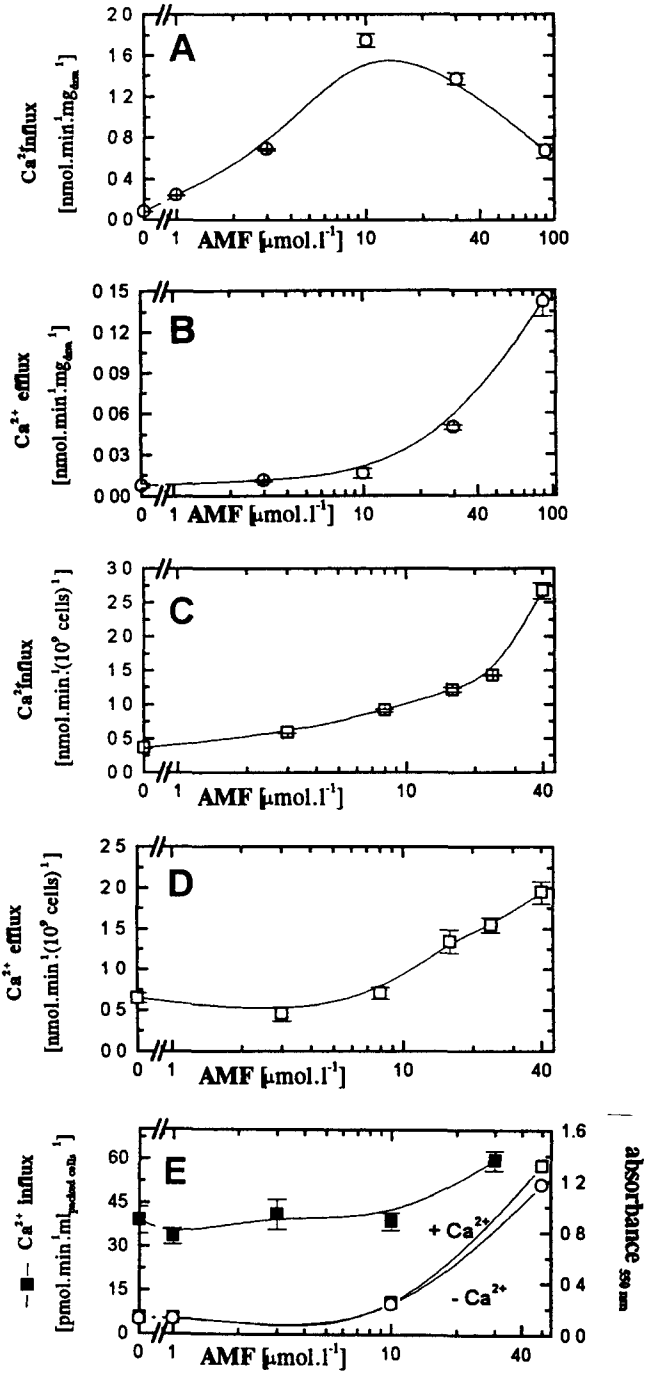

Figure 1. The effect of azalomycin F on $^{45}Ca^{2+}$ influx (A,C) and $^{45}Ca^{2+}$ efflux (B, D) in *T. viride* submerged mycelium (A, B), *S. cerevisiae* cells, (C, D), and on $^{45}Ca^{2+}$ influx and hemolysis in human red cells (E). The $^{45}Ca^{2+}$ influx and $^{45}Ca^{2+}$ efflux were measured as described in Materials and Methods using concentrations of AMF as indicated and 0.2% (v/v) methanol (as a solvent for AMF) Representative of three independent experiments each performed in triplicates. The $^{45}Ca^{2+}$ influx in red cells and hemolysis (E) were measured as described in Materials and Methods.

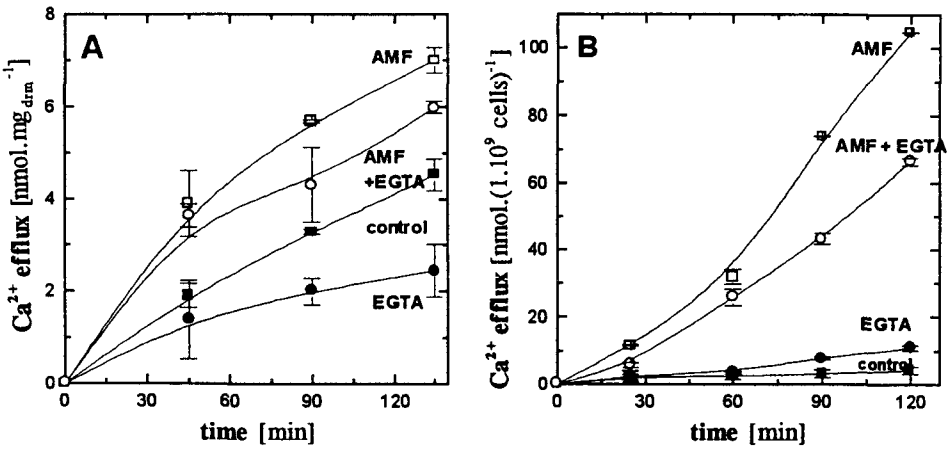

Figure 2. The effect of EGTA on the AMF-induced $^{45}Ca^{2+}$ efflux from *T viride* submerged mycelium (A) and from *S cerevisiae* cells (B) The $^{45}Ca^{2+}$ efflux was measured as described in Materials and Methods with 10 mmol/l EGTA added prior to the start of measurement (the same volume of water was added in controls) The values were corrected for the radioactivity measured at time zero Representative of two experiments

On the other hand, there was no effect of this compound on the movement of $^{45}Ca^{2+}$ across the human red blood cell membrane (Fig. 1E). AMF concentrations as high as 10 μmol/l did not induce hemolysis of red blood cells, neither did they stimulate the $^{45}Ca^{2+}$ influx (Fig. 1E), although at higher concentrations (e.g., 40 μmol/l) a stimulation of hemolysis was observed

Furthermore, AMF had no effect on the transfer of $^{45}Ca^{2+}$ across the water/organic solvent (*n*-octanol) interface (data not shown). It seems unlikely that these results could be explained by a Ca^{2+} ionophoric activity of this compound. Desertomycin, an antibiotic with a similar structure, stimulated $^{45}Ca^{2+}$ uptake in the same concentration range (data not shown).

In *T. viride* mycelium (Fig. 2A) and in *S cerevisiae* cells (Fig. 2B), the stimulatory effect of AMF on the efflux of $^{45}Ca^{2+}$ was slightly lowered by ethyleneglycol-bis-(β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA). In the absence of AMF, EGTA markedly inhibited $^{45}Ca^{2+}$ efflux in *T viride* but slightly stimulated it in *S. cerevisiae*. 1,2-bis(2-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) (10 mmol/l), surprisingly, did not inhibit the effect of AMF (not shown).

Effect of AMF on $^{45}Ca^{2+}$ fluxes in isolated subcellular fractions

Specific activities of the organellar marker enzymes in HOF and LOF from *T viride* are shown in Table 1. Cytochrome c oxidase and α-D-mannosidase were concentrated in HOF, while NADPH: cytochrome c oxidoreductase and vanadate-sensitive H^+-ATPase in LOF, indicating that the mitochondria and vacuoles were

Table 1. The distribution of marker enzymes in the heavy (HOF) and light (LOF) organellar fractions isolated from *S cerevisiae* cells and from the submerged *T viride* mycelium

	Enzyme	Cytochrome c oxidase	NADPH cyt C Oxido-reductase	α-D-manosidase	GDPase	H^+-ATPase
Strain	Fract	Specific activity [nmol min^{-1} mg $prot^{-1}$]				
Saccharomyces	LOF	0	11 62±2 19	0 17±0 08	17 04±0 65	92 49±3 71
cerevisae	HOF	11 45±1 01	20 59±0 43	3 17±0 09	33 73±0 76	127 04±2 36
Trichoderma	LOF	0 40±0 19	2 20±0 24	1 11±0 27	33 60±0 85	87 23±1 38
viride	HOF	10 86±0 41	1 34±0 06	2 27±0 13	27 80±0 47	45 99±0 82

Data from representative of three experiments performed in triplicates Enzyme activities and protein concentrations were measured as described in Materials and Methods

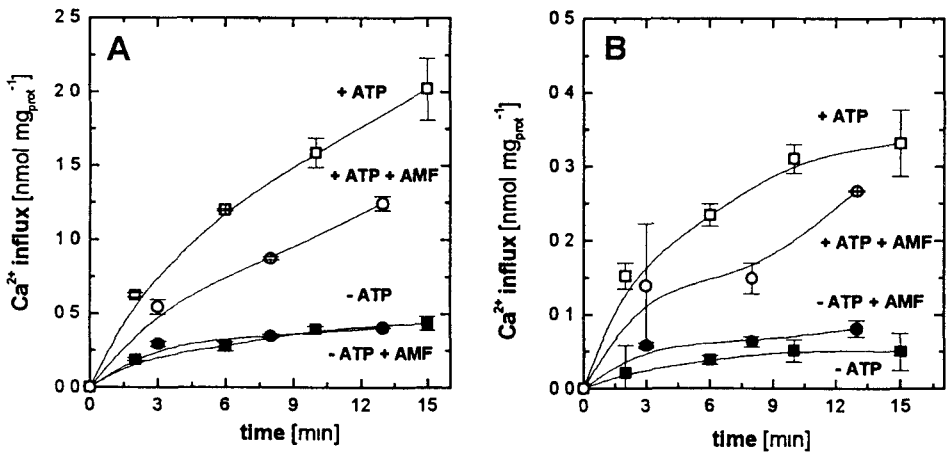

Figure 3. The effect of AMF on the ATP- dependent and ATP-independent $^{45}Ca^{2+}$ uptake in the heavy (A) and light (B) organellar fractions isolated from *T viride* submerged mycelium The $^{45}Ca^{2+}$ uptake was measured as described in Materials and Methods in the presence of 1 mmol/l ATP (open symbols) or without ATP (closed symbols) in controls AMF (30 μmol/l) (circles) or 0 2% methanol (squares) were added 5 min before the addition of ATP Addition of $^{45}Ca^{2+}$ followed the addition of ATP and aliquots were withdrawn at the time indicated The values for the ATP-dependent $^{45}Ca^{2+}$ uptake were corrected for the radioactivity measured at time zero and for the radioactivity in controls without ATP Representative of three experiments

concentrated in HOF and the endoplasmic reticula and the plasma membranes in LOF The specific activity of GDPase, a marker of Golgi apparatus was distributed approximately equally in LOF and HOF. Both fractions took up $^{45}Ca^{2+}$ in ATP-dependent manner (Fig 3) The ATP-dependent $^{45}Ca^{2+}$ uptake in HOF and LOF

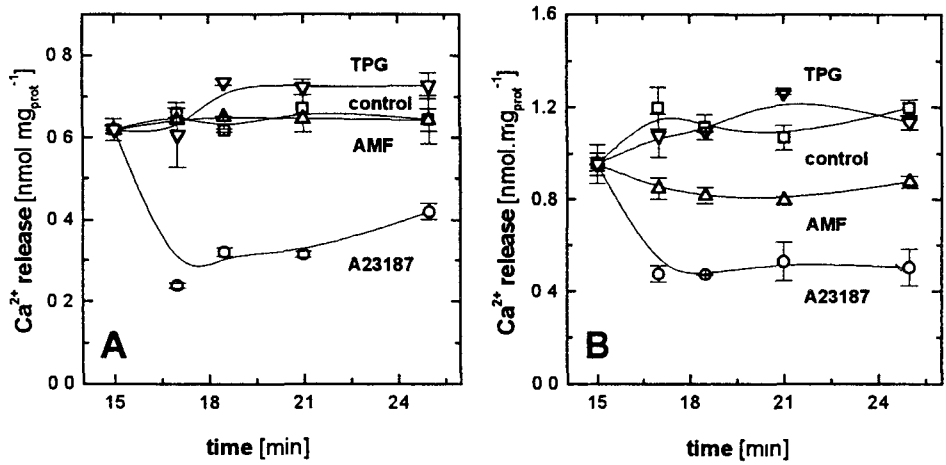

Figure 4. The effects of AMF, TPG and A23187 on $^{45}Ca^{2+}$ release from the heavy (A) and light (B) organellar fractions isolated from *T. viride* submerged mycelium. Organellar suspensions from *T. viride* mycelium were loaded with $^{45}Ca^{2+}$ in ATP-dependent manner (1 mmol/l ATP) for 15 min. Tested substances: 30 μmol/l AMF (triangles), 10 μmol/l TPG (inverted triangles), 15 μg/ml A23187 (circles), 0.2% methanol (squares) were added to the suspension. Aliquots were withdrawn and immediately filtered as described in Materials and Methods. Representative of four experiments.

was inhibited by AMF (30 μmol/l), whereas the residual $^{45}Ca^{2+}$ uptake observed without ATP was insignificantly influenced by AMF (Fig. 3).

The subcellular fractions were pre-loaded with $^{45}Ca^{2+}$ in ATP-dependent manner and the effects of AMF, A23187 and thapsigargin (TPG) on the release of $^{45}Ca^{2+}$ were examined. A23187 caused $^{45}Ca^{2+}$ to be released from both HOF and LOF, however, AMF caused the release from LOF only (Fig. 4). On the other hand, TPG, a compound which releases Ca^{2+} from microsomes of animal cells due to its inhibitory action on the Ca^{2+}-ATPase, was inactive in both subcellular fractions. Ryanodine (10 μmol/l) had no effect on the Ca^{2+} release (not shown).

HOF and LOF isolated from *S. cerevisiae* displayed slightly different patterns of marker enzyme distribution (all enzymes were more concentrated in HOF) and showed also the ATP-dependent $^{45}Ca^{2+}$ uptake which exceeded the ATP-independent one by a factor more than 7. $^{45}Ca^{2+}$ accumulated by this way could be released by AMF from LOF and slightly from HOF (Fig. 5). A23187 released $^{45}Ca^{2+}$ from both subcellular fractions, and TPG had only a negligible effect on HOF and a biphasic effect on LOF (Fig. 5).

Changes in $[Ca^{2+}]_{cyt}$ in yeast cells measured by aequorin luminescence

AMF induced an immediate and rapid aequorin luminescence indicating $[Ca^{2+}]_{cyt}$ elevation of *S. cerevisiae* containing aequorin in the cytoplasm (Fig. 6). A gradual

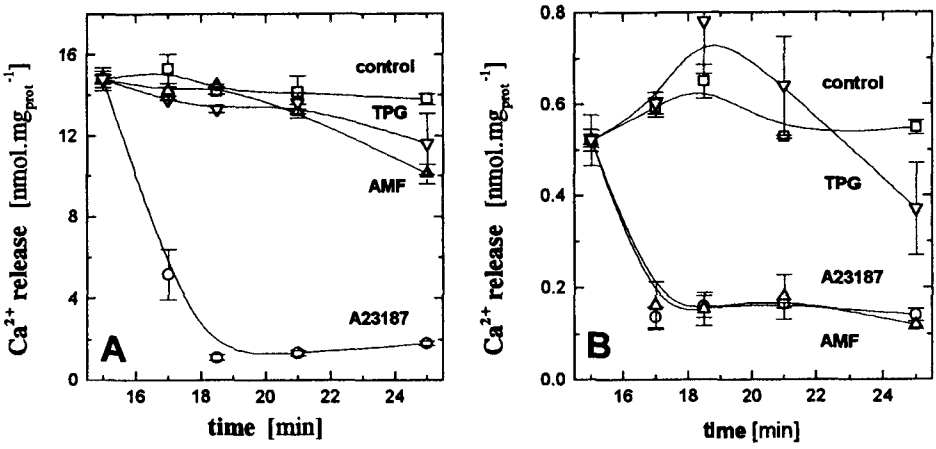

Figure 5. The effects of AMF, thapsigargin and A23187 on $^{45}Ca^{2+}$ release from the heavy (A) and light (B) organellar fractions isolated from the *S. cerevisiae* Organellar suspensions from *S cerevisiae* were loaded with $^{45}Ca^{2+}$ in ATP-dependent manner (1 mmol/l ATP) for 15 min Tested substances (30 μmol/l AMF (triangles), 10 μmol/l TPG (inverted triangles), 15 μg/ml A23187 (circles), 0 2% methanol (squares) were added to the suspension Aliquots were withdrawn and immediately filtered as described in Materials and Methods Representative of two experiments

small $[Ca^{2+}]_{cyt}$ elevation was induced by 25 μmol/l AMF. A rapid and transient $[Ca^{2+}]_{cyt}$ elevation was observed with 50 μmol/l AMF. The $[Ca^{2+}]_{cyt}$ elevation was dependent on AMF concentrations, higher concentrations induced quicker and stronger responses.

Discussion

The above results show that AMF perturbs the homeostasis of Ca^{2+} in Deuteromycete *Trichoderma viride* and in Ascomycete *Saccharomyces cerevisiae.* The fact that this agent is able to stimulate both Ca^{2+} influx and efflux, and taking into account that there is no evidence for its ionophoric action, could lead to the proposal that the primary site of its action is stimulation of Ca^{2+} influx which, due to the increase of $[Ca^{2+}]_{cyt}$, activates the extracellular efflux and/or organellar sequestration of Ca^{2+}.

The stimulation of Ca^{2+} influx by AMF in intact cells could be explained by the direct stimulation of the putative transport protein participating in the Ca^{2+} influx, e.g., by changes in membrane potential, or by the direct interaction. For this explanation we were unable to present direct evidence in *T. viride* as the transport system conveying non-stimulated Ca^{2+} influx has been described only phenomenologically so far (Kryštofová et al. 1995; Šimkovič et al. 1997), and no molecular or genetical tools are available to test this possibility. In *S. cerevisiae*,

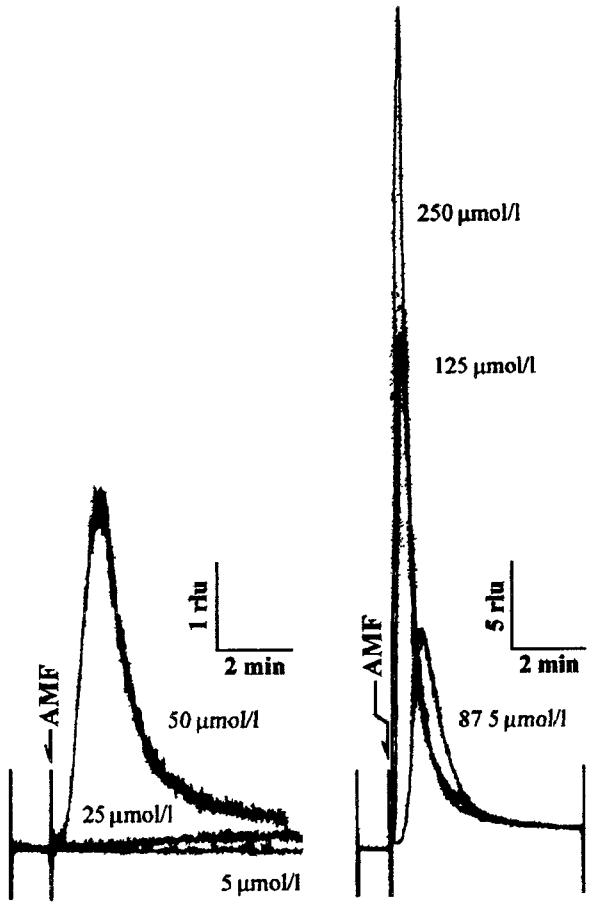

Figure 6. AMF-induced aequorin luminescence reflecting $[Ca^{2+}]_{cyt}$ elevation in *S cerevisiae* Indicated concentrations of AMF were added at the time indicated by the arrows and aequorin luminescence was recorded Total reaction volume was 200 μl

although the properties of the Ca^{2+} influx were thoroughly described previously (Roomans et al 1979, Eilam 1984) and several genes were found which are involved in Ca^{2+} homeostasis (Beeler et al 1994, Ono et al 1994, Takita et al 1995, Pozos et al 1996, Tanida et al 1996, Tsuchiya et al 1996, Paidhungat and Garrett 1997, Fischer et al 1997, Durr et al 1998), none of them was shown to encode the protein conveying the non-stimulated Ca^{2+} influx across the plasma membrane

The stimulation of the $^{45}Ca^{2+}$ efflux upon the AMF treatment in both tested microorganisms could be explained by increasing $[Ca^{2+}]_{cyt}$ which, consequently, activates the outward-directed Ca^{2+} extrusion and/or organellar sequestration of Ca^{2+} This was directly demonstrated in the aequorin-expressing *S cerevisiae*

(Fig 6) The partial inhibition of the AMF-induced $^{45}Ca^{2+}$ efflux with EGTA (Fig 2) is in accordance with the notion that there is Ca^{2+}/Ca^{2+} exchange across the surface membrane under these conditions

In fact, AMF perturbed also Ca^{2+} movements in microsomal vesicles Its effect on the $^{45}Ca^{2+}$ release from microsomal vesicles (Figs 4, 5) could be due to the stimulation of the Ca^{2+} channel activity with functional similarity to ryanodine receptor or inositol 1,4,5-trisphosphate receptor, or the inhibition of the organellar Ca^{2+}-ATPase activity (thapsigargin-like effect) (Manoach et al 1999) Results obtained with *Trichoderma* vesicles (Figs 3, 4) better fit the notion that AMF affects both Ca^{2+}-influx and -efflux pathways which maintain the organellar Ca^{2+} homeostasis Surprisingly, thapsigargin (TPG), an agent which is known to release Ca^{2+} from organelles isolated from animal cells, was without effect on the Ca^{2+} release from *T viride* vesicles and had only a minor, if any, effect on *S cerevisiae* vesicles (Fig 5) suggesting that the molecular determinants of the putative TPG target protein (presumably the microsomal Ca^{2+}-ATPase) involved in its interaction with TPG in *Trichoderma* or *Saccharomyces* are different from those in animal cells The possibility that this protein is absent in these microorganisms seems to be unlikely as the organelles take up Ca^{2+} in an ATP-dependent manner (Fig 3) The distribution of marker enzymes in the isolated subcellular fractions from *T viride* and *S cerevisiae* (Table 1) did not show which organelle is the target Ca^{2+} store for AMF action

The overall effect of AMF on the $^{45}Ca^{2+}$ fluxes across the cytoplasmic membrane and the intracellular Ca^{2+} stores phenomenologically conform to the concept of the capacitative Ca^{2+} entry This concept, originally developed in order to explain the ligand-receptor-induced effects on the Ca^{2+} homeostasis in animal cells (Putney 1986), postulated that the Ca^{2+} influx is activated after the depletion of the cytoplasmic Ca^{2+} store which is mediated by an unknown messenger On the other hand, Kiselyov et al (1998) suggested that the capacitative Ca^{2+} influx could be mediated also *via* direct interaction of membranes, i e , without the involvement of second messenger However, evidence which would unequivocally support the capacitative mechanisms is still lacking (Shuttleworth 1999, for review)

One of the questions emerging from our results is whether they could present evidence for the capacitative Ca^{2+} influx mechanism In this respect, the presented results conform with both the capacitative Ca^{2+} entry model and also the model of the direct activation of the Ca^{2+} influx pathway

In summary, novel effects of AMF on the fungal Ca^{2+}fluxes were demonstrated which involve both the cytoplasmic membrane and intracellular Ca^{2+} stores Hopefully, AMF could become a useful tool for a better understanding of the Ca^{2+} homeostasis in fungi

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