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EXPERIMENTAL ARTICLES

Analysis of the Ca^{2+} Response of Mycelial Fungi to External Effects by the Recombinant Aequorin Method

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Abstract—Using the mutant strain *Aspergillus awamori* 66A, producing the recombinant Ca^{2+}-dependent photosensitive protein aequorin, the dynamics of Ca^{2+} was studied for the first time in the cytosol of micromycetes exposed to stressful factors, such as an increase in extracellular Ca^{2+} to 50 mM, hypoosmotic shock, and mechanical shock. The cell response to stress proved to involve an increase in the Ca^{2+} concentration in the cytosol, which was determined from the amplitude of aequorin luminescence and the time of the amplitude enhancement and relaxation. The level of the Ca^{2+} response depended on the physiological stimulus. Inhibitory analysis with various agents that block Ca^{2+} channels and with agonists that specifically enhance the activity of the channels suggested that (1) the level of Ca^{2+} in the cytosol of micromycetes increases in response to stress because of the ion influx from both the growth medium and intracellular reservoirs and (2) potential-dependent transport systems play the major role in the Ca^{2+} influx into the cytosol of the micromycete cells.

Key words: stress, mycelial fungi, *Aspergillus awamori*, recombinant aequorin, Ca^{2+} dynamics.

The concepts of secondary messengers that regulate cell metabolism emphasize the universal role of calcium [1]. Ca^{2+} is the messenger involved in the stress response, which is confirmed by the following: (1) cell response to some changes in the ambient medium is accompanied by a primary increase in the intracellular calcium concentration; (2) blockage of Ca^{2+} influx into cells inhibits cell response; and (3) stimulation of processes that lead to enhancement of the intracellular Ca^{2+} concentration increases the intensity of the stress response [2].

In eukaryotes, intracellular Ca^{2+} pool formation is involved in regulatory processes and proceeds via two pathways: calcium is transported from the ambient environment or from cellular organelles that are reservoirs of these ions. In both cases, calcium influx into the cytosol occurs via special channels in the plasma membrane and cell organelle membranes. Much has been reported on the Ca^{2+} channels in mammals and plants [3–5] but not in mycelial fungi, which is in part caused by distinctions in the architectonics of lower and higher eukaryote cells.

Ca^{2+} dynamics in a cell and specific functioning of Ca^{2+} channels are mostly assessed from differences in membrane potential and by using isotopic analysis of the transported Ca^{2+} under conditions that change the functioning of Ca^{2+} channels. The method of measuring free calcium ion concentration using aequorin, a Ca^{2+}-dependent photosensitive protein isolated from *Aequorea victoria*, also provides good results [6, 7]. However, it is difficult to introduce aequorin into cells, and, therefore, a genetic construction has been obtained that ensures aequorin expression in recipient cells, which is a great advance in studies of calcium metabolism [8]. The mechanisms that regulate the level of Ca^{2+} in a cell have been analyzed by various methods; nevertheless, this problem remains insufficiently studied and important in modern physiology. Mycelial fungi are a good model for clarification of the regulatory role of Ca^{2+} in cell adaptation because these microorganisms are labile in response to stressful action [9].

In this work, the effects of various physiological factors on the dynamics of the intracellular Ca^{2+} concentration were studied in *Aspergillus awamori.*

MATERIALS AND METHODS

The subject of this study was the strain *Aspergillus awamori* 66A, obtained from the Laboratory of Micromycete Cells, Edinburgh University. This is a mutant strain in which the photoprotein aequorin is expressed [8].

Interaction of the photoprotein aequorin with Ca^{2+} results in aequorin cleavage to apoaequorin and coelenteramide, which is accompanied by the emission of energy in the form of blue light. The luminescence intensity is proportional to the Ca^{2+} concentration in the

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Table 1. Dynamics of intracellular Ca^{2+} in cells of *A. awamori* exposed to physiological stressful impacts

Impact	Parameters of Ca^{2+} response	
	Ca^{2+} concentration, μM	Duration, s*
Extracellular Ca^{2+}, mM:		
0.05	0.20 ± 0.070	49–51
5.50	0.80 ± 0.069	24–26
Hypoosmotic shock	0.20 ± 0.011	39–40
Mechanical stimulation	0.15 ± 0.009	48–50

Note: Mean values of five measurements are shown.
* Duration of Ca^{2+} response, $A = 1/2\,A_{max}$, s.

cytosol. Aequorin can be restored by incubation of apoaequorin with the chromophore coelenterazine. The emission of light is weak, and, therefore, a special luminometer (EG&G Berthold LB96P Microlumat) equipped with a photomultiplier was used.

The culture of *A. awamori* 66A was grown at 30°C in liquid Vogel medium [10] supplemented with 1% sucrose in a 96-well plate. Each well contained 100 μl of spore suspension with an initial concentration of 5×10^5 cells/ml. At a certain growth phase, the micromycete culture was incubated for 4 h with coelenterazine to restore aequorin. Coelenterazine was dissolved in 40 μl of methanol and immediately introduced into each of the wells to a final concentration of 2.5 μM. After aequorin restoration, tested compounds dissolved in Vogel medium were introduced into the wells or the micromycete culture was exposed to stressful impact, and light emission was then measured on a luminometer, being expressed in relative light units (RLU).

The intracellular concentration of Ca^{2+} was calculated for the luminescence value using the empirically derived formula

$$pCa = 0.332588(-\log k) + 5.5593,$$

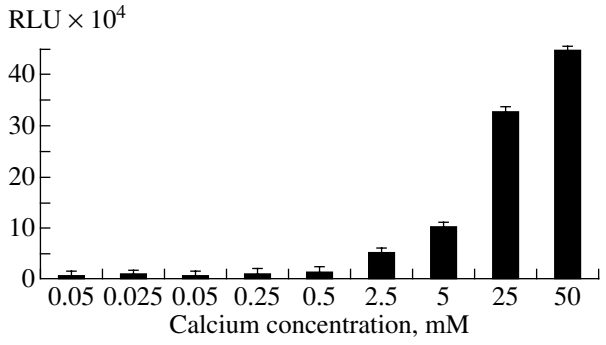

Fig. 1. Effect of exogenous $CaCl_2$ added at various concentrations on Ca^{2+} cell response (aequorin luminescence) in a 48-h culture of *A. awamori*. RLU, relative light units.

where k is the ratio of luminescence at a given moment to the total maximum luminescence [11]. Total maximum luminescence was defined as the value measured after total degradation of cell aequorin caused by the addition of Ca^{2+} to the cell suspension to a final concentration of 1.5 M.

The physiological stimuli employed were those tolerated by *A. awamori*. High Ca^{2+} concentrations were reached by additional introduction of $CaCl_2$ into the Vogel medium (to final concentrations from 0.005 to 50 mM). Osmotic shock was imposed by threefold dilution of the medium; mechanical shock was imposed by culture agitation on a shaker (140 rpm) for 5 min. All experiments were run in five replicates. The results were processed statistically using variation statistics analysis and the significance level $P < 0.05$.

RESULTS AND DISCUSSION

The dynamics of the Ca^{2+} concentration in the cytosol of *A. awamori* cells subjected to various physiological stimuli (stressful factors), such as high concentrations of extracellular Ca^{2+}, hypoosmotic shock, and mechanical impact (agitation of a cell suspension), was studied by the recombinant aequorin luminescence method.

In the first series of experiments, the micromycete cell response was determined during the exponential growth phase (48 h) from the amplitude of luminescence (A) recalculated to the concentration of intracellular Ca^{2+}. The results are shown in Table 1. Note that the level of intracellular Ca^{2+} grew for about 3 s irrespective of the nature of the physiological stimulus. Ca^{2+} response was determined as dependent on the amount of inducing substances. For example, the additional introduction of Ca^{2+} ions ($CaCl_2$) into the growth medium within the range 0.005–0.5 mM had no effect on the intracellular calcium ion concentration. A further increase in the content of Ca^{2+} in the medium from 0.5 to 50 mM led to exponential growth of the intracellular level of these ions (Fig. 1).

The cell calcium response depended not only on the intensity of the stressful impact but also on the physiological age of the micromycete (Fig. 2). The response of actively growing cultures (24 and 48-h) to a $CaCl_2$ concentration in the medium from 0.5 to 5.0 mM was much weaker than that of stationary cultures (72 h), where the same Ca^{2+} concentrations strongly stimulated intracellular accumulation of these ions. In the latter variants, the light emission was as high as $(0.8–1.0) \times 10^6$ and even 1.2×10^6 RLU in some experiments, which allowed luminescence to be observed with the unaided eye. In cells of the later stationary phase (96 h), the Ca^{2+} response declined again (6- to 12-fold). This may be partially explained by changes in the absorption capacity of these cells, which became pigmented. However, the main reason for the decline in the response is that, in late-stationary cells, the intracellular Ca^{2+} is

mostly bound rather than free because it stabilizes cell biopolymers and supramolecular structures and, therefore, is not detectable by the method used in this work. All these factors may account for the decrease in aequorin luminescence, which indicates the decrease of the Ca^{2+} response of fungal cells in the poststationary phase of their growth. Note that, in spores and sporulating cells of both pro- and eukaryotic microorganisms, the total amount of Ca^{2+} is known to increase as compared to growing cells and cells of the stationary growth phase. However, in resting forms, Ca^{2+} is present in the bound state [9, 12].

When determined by the recombinant aequorin method, the dynamics of Ca^{2+} in cells was evaluated from the amplitude of luminescence and the time of amplitude increase and relaxation. This makes it possible to compare the cell stress response to various factors. The highest amplitudes of luminescence were observed when an excess of $CaCl_2$ was present in the medium. The effect of 5.0 mM $CaCl_2$ on *A. awamori* resulted in a higher amplitude and a lower time of relaxation than the presence of 0.5 mM $CaCl_2$ (Fig. 3). Osmotic shock caused a higher amplitude of luminescence than mechanical treatment did (Fig. 4), and the rate of Ca^{2+} accumulation in these two cases was higher than during Ca^{2+} response to the presence of 0.5 mM exogenous $CaCl_2$. The longest period of relaxation was observed after mechanical impact and osmotic shock (Fig. 4).

The differences in the parameters of Ca^{2+} responses to different external impacts (Table 1; Figs. 3, 4) may suggest that different ion-transporting systems account for these responses. To verify this suggestion, inhibitory analysis was employed. We used several pharmacological preparations known to inhibit different calcium channels in animal and plant cells [13, 14]. The effect of inhibitors on the Ca^{2+} response was studied under the action of various physiological stimuli on cells of a growing fungal culture (48 h); the results were expressed as the amount of an inhibitor (mM) that reduced Ca^{2+} accumulation by 50% as compared to the control variants, without the addition of any inhibitors (Table 2).

Ca^{2+} channel–blocking agents, such as La^{3+} (lanthanum) and KP4, inhibited the Ca^{2+} response of micromycetes to hypoosmotic shock and the increase in the level of intracellular Ca^{2+} but failed to have any effect in case of mechanical cell treatment. An inhibitory effect of the divalent cation BAPTA was observed under all stressful impacts.

The La^{3+} action was studied in detail. Lanthanum is known to block L-type calcium channels; at concentrations of 10–20 mM, this agent inhibited accumulation of intracellular Ca^{2+} in response to a stress-induced increase in the concentration of exogenous Ca^{2+} and in the case of hypoosmotic shock (Fig. 5; Table 2). The same agent had no effect on cells of a growing culture (24 h) exposed to mechanical stress (Table 2).

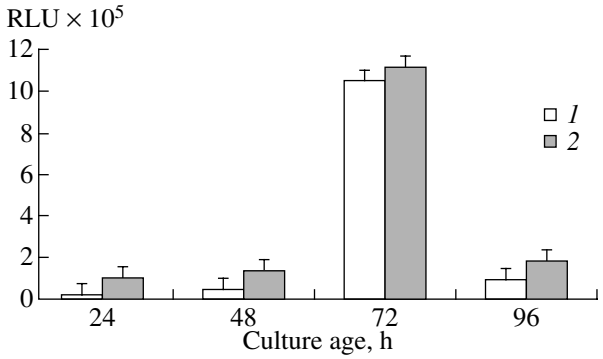

Fig. 2. Dependence of *A. awamori* Ca^{2+} response (aequorin luminescence) on physiological age of the micromycete exposed to the action of $CaCl_2$: (*1*) 0.5 and (*2*) 1.5 mM Ca^{2+}.

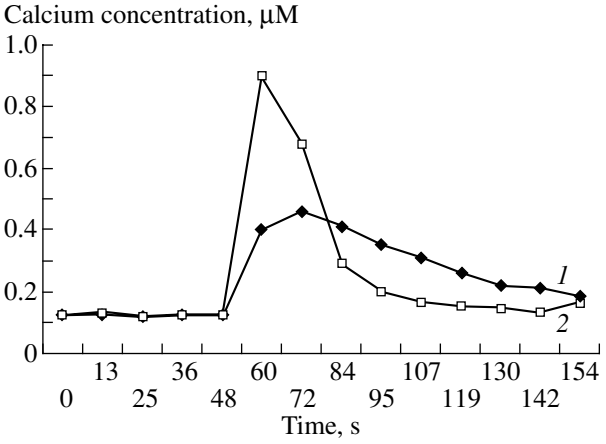

Fig. 3. Dynamics of Ca^{2+} ions in 24-h *A. awamori* cells exposed to the action of $CaCl_2$: (*1*) 0.5 and (*2*) 5 mM of Ca^{2+}.

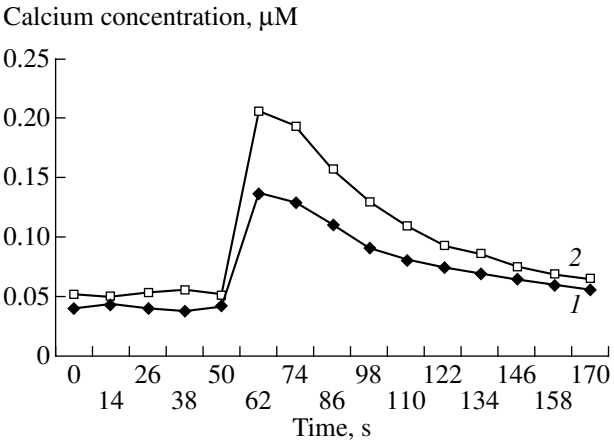

Fig. 4. Dynamics of Ca^{2+} ions in 24-h *A. awamori* cells exposed to (*1*) mechanical stress and (*2*) hypoosmotic shock.

Table 2. Effect of inhibitors of Ca^{2+} transport on intracellular Ca^{2+} concentration in *A. awamori* exposed to stressful impacts

Inhibitor	Impact	IC_{50}, mM*		
		Extracellular Ca^{2+}	Mechanical stimulus	Hypoosmotic shock
Lanthanum chloride ($LaCl_3 \cdot 7H_2O$)	L-type agent blocking Ca^{2+} channels	10–14	–**	10–14
KP4	Inhibits Ca^{2+}-channel activity in *N. crassa*	0.2–0.5	–	0.2–0.5
Ryanodine (a natural alkaloid)	Stimulates Ca^{2+} release from intracellular organelles	–	–	–
Nifedipine (from the group of dihydropyrines)	L-type agent blocking Ca^{2+} channels	–	–	–
TMB-8 [8-*N,N*-diethylamine-octyl-3,4,5-trimethoxybenzoate]	Blocks Ca^{2+} release from intracellular organelles	–	–	–
Verapamil (hydrochloride)	L-type agent blocking Ca^{2+} channels	–	–	–
BAPTA [1,2-bis(*o*-amino-5-bromphenoxy)ethane-*N,N,N',N'*-tetraacetic acid, 4Na]	Binds Ca^2+ ions, which become inaccessible for a cell	IE***	0.5–5.0	0.5–5.0

Note: Mean value of five measurements is shown in each case.
* IC_{50}, the concentration that decreases the intracellular level of Ca^{2+} by 50% of the maximum level in the control without inhibitor.
** No inhibition.
*** Incorrect employment.

Corzo and Sanders [15] reported low La^{3+} concentrations (lower than 5 mM) to cause membrane depolarization in *Neurospora crassa* and, consequently, to change the membrane functional activity, which nonspecifically affects the influx of Ca^{2+} into the cytoplasm through the cytoplasmic membrane and the membranes of cell organelles (Ca^{2+} reservoirs). Higher La^{3+} concentrations inhibit Ca^{2+} transport specifically. In our experiments with fungi, the amplitude of aequorin luminescence decreased with increasing lanthanum concentrations. The magnitude of the effect of La^{3+} apparently depends on the state of the membrane. Indeed, although at concentrations of 10–20 mM La^{3+} reduced the amplitude of luminescence in growing *A. awamori* cells, the same La^{3+} concentrations only slightly increased the level of intracellular Ca^{2+} in lag-phase cells (it should be recalled here that physiological stimuli caused no Ca^{2+} response in lag-phase *A. awamori* cells). The discovered patterns of Ca^{2+} response and the dependence of the effect of La^{3+} on the physiological age of the micromycete and, thus, on the peculiarities in the cell membrane structure are not at odds with concepts of the physiology of microbial growth and development. For example, it has been shown that directed changes in the phase state of membranes affect the rate of germination of resting forms [16].

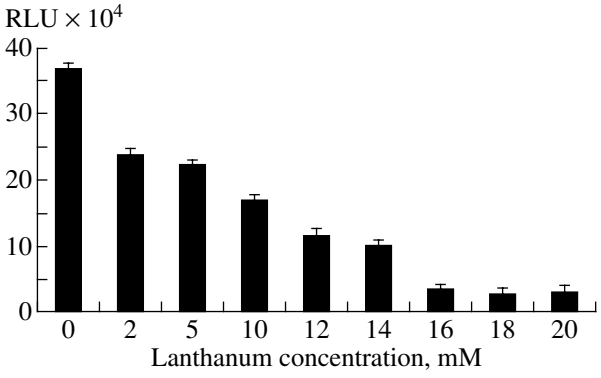

Fig. 5. Dependence of the Ca^{2+} response of *A. awamori* cells exposed to the action of exogenous $CaCl_2$ (50 mM) on the lanthanum chloride concentration.

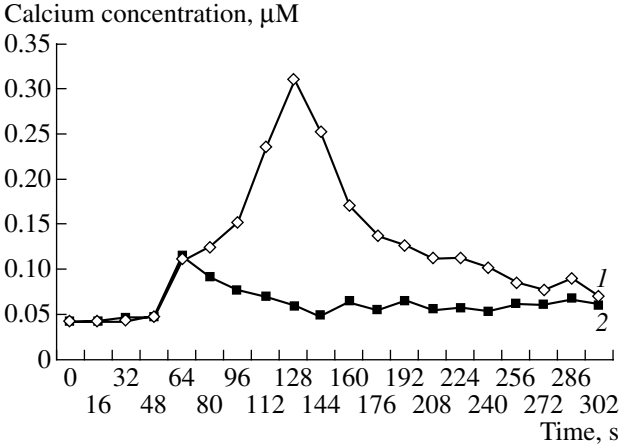

Fig. 6. Effect of caffeine (10 mM) on the Ca^{2+} response of *A. awamori* cells exposed to a mechanical stimulus: (*1*) caffeine added; (*2*) control.

Table 3. Effect of agonists on the dynamics of intracellular Ca^{2+} in *A. awamori* exposed to mechanical action

Ca^{2+} agonist	Mechanism of action	Increase in Ca^{2+} concentration	Minimum effective concentration
A23187 (calcimycin)	Ionophore relatively specific for Ca^{2+}	+	1 μM
Br-A23187 (brom-calcimycin)	Mobile Ca^{2+} carrier	+	10 μM
BAY (3-[methylphenylsulfonyl]-2-propenennitrile)	Causes Ca^{2+} release from intracellular organelles	–	IE*
Caffeine	Causes Ca^{2+} release from plasma reticulum	+	5 mM
Cyclopiazonic acid (isolated from cells of *Penicillium cyclopium*)	Inhibitor of Ca^{2+}-ATPases; causes changes in membrane potential	+	20 μM

Note: Mean value of five measurements is shown in each case.
* Incorrect employment.

On the transition of microbial cultures from the exponential to the linear growth phase, endogenous membranotropic agents cause uncoupling of respiration and oxidative phosphorylation in membranes [17].

Some other inhibitors of calcium transport—nifedipine, TMB-8, diethylaminooctyl methoxybenzene, verapamil, and ryanodine, the effects of which have been studied in detail in mammalian cells—were tested in micromycetes for the first time. These compounds did not produce a statistically significant effect on the increase in the intracellular Ca^{2+} concentration induced by external factors in lower eukaryotes.

Thus, some compounds known to inhibit calcium transport systems in multicellular organisms were found in this work to have an effect on the Ca^{2+} response of mycelial fungi exposed to stressful factors. The effects of these compounds depended on the physiological stimulus and did not necessarily coincide with the inhibitory effects previously described for cells of higher eukaryotes. Thus, none of the tested inhibitors that block the release of Ca^{2+} from the reservoirs of the endoplasmic reticulum in multicellular organisms had any effect on the Ca^{2+} response of *A. awamori.* This suggests that the major role in the micromycete stress response is played by the potential-dependent systems of Ca^{2+} transport located in the cytoplasmic membrane, whose structure is different in lower and higher eukaryotes. However, it should not be ruled out that the intracellular Ca^{2+} reservoirs may play a role in the stress response of fungal cells, but this is difficult to show by inhibitory analysis because inhibitors of the calcium channels of the organelle plasmalemma of higher eukaryotes are inefficient in fungi.

The Ca^{2+} transport systems of micromycetes were additionally studied using agonists, agents that enhance calcium channel activity (Table 3). Except for BAY, all tested agonists (caffeine, cyclopiazonic acid, and calcimycin and its bromated derivative) increased the amplitude of luminescence and, thus, stimulated intracellular Ca^{2+} accumulation in cells of *A. awamori* (Table 3).

Cyclopiazonic acid and caffeine are known to induce a release of Ca^{2+} from organelles into the cytosol. Hence, it can be inferred that, in mycelial fungi, certain mechanisms control translocation of the accumulated intracellular Ca^{2+} into the cytosol under stressful conditions. Of interest is the fact that, in the case of mechanical stress, the caffeine produced a biphasic effect on the Ca^{2+} (Fig. 6). The first peak of luminescence resembled the response to mechanical stress in the control without caffeine. The second maximum, the amplitude and relaxation time of which were significantly greater, might be a result of caffeine-induced Ca^{2+} release from intracellular organelles, suggesting that both extracellular and intracellular Ca^{2+} reservoirs are switched on by the same regulatory system and that special mechanisms control coordinated Ca^{2+} transport into the cytosol of micromycete cells.

Thus, our results suggest that the cells of mycelial fungi have several systems that control the transport of Ca^{2+} and a stable level of this ion in the cytosol during cell response to stress. The results of inhibitory analysis show that calcium influx from the growth medium or intracellular reservoirs is mostly ensured by potential-dependent systems. The level of Ca^{2+} in the cytosol is determined by the balance of Ca^{2+} influx, efflux, and accumulation in reserve cell organelles and by Ca^{2+} binding to proteins and some other cellular components. Although these processes are separate, their cofunctioning seems to ensure fine regulation of the level of Ca^{2+} in the cell cytosol. On the other hand, the above processes may be regulated by a common mechanism, and this problem will be considered in our forthcoming publication.

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