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EXPERIMENTAL ARTICLES

Effect of a Chemical Analogue of Autoinducers of Microbial Anabiosis on the Ca^{2+} Response of Mycelial Fungi

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Abstract—The microbial alkylhydroxybenzenes (AHB), which are anabiosis autoinducers also termed d_1 factors, participate in the stress response of mycelial fungi, as determined from changes in intracellular Ca^{2+} concentration. By using the genetically modified strain *Aspergillus awamori* 66A, which produces the recombinant Ca^{2+}-dependent protein aequorin, the dynamics of Ca^{2+} was studied in the cytosol of cells exposed to mechanical shock in the presence of protective doses (0.001–0.01% w/vol) of a chemical AHB analogue, 4-*n*-hexylresorcinol. As under stressful conditions, Ca^{2+} concentration increases in the cell cytosol in response to an enhanced AHB level in a growing fungal culture; thus, AHB is perceived by cells as a stress signal. The level of cell response, which was determined from the amplitude of luminescence dependent on the Ca^{2+} concentration in the cytosol, was related to the physiological age of the cells and the AHB concentration. Micromycete preincubation with AHB was found to protect cells from subsequent stress; this was reflected in the Ca^{2+} response. The protective AHB effect was manifested as (1) a significant decrease in the amplitude of luminescence and, thus, in Ca^{2+} accumulation in the cytosol during subsequent mechanical stress (as compared to the control—mechanical stress only); (2) development of a secondary Ca^{2+} response, which was not observed in the control; and (3) a high level of Ca^{2+} retained in the cytosol for a long time in the presence of AHB (as compared to the control without preincubation with AHB). The mechanisms underlying the AHB effect on Ca^{2+} transport systems are discussed.

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

Changes in intracellular Ca^{2+} concentration are known to play an important role in the regulation of all physiological processes occurring in the cell: growth, division, excitation, secretion, development of microbial resting forms, and apoptosis in multicellular organisms. An enhanced Ca^{2+} concentration in cells caused by changes in calcium regulation is a specific feature of oxidative stress, which develops in response to nearly all stressful impacts [1].

In eukaryotic cells, the Ca^{2+} pool in the cytosol is formed due to the influx of these ions from the growth medium and from cellular organelles, the reservoir of calcium. In both cases, Ca^{2+} is transported through the membrane calcium channels and this process depends on the structural and functional state of membranes and channel proteins.

It was of interest to study in stress-exposed microbial cells the dependence of intracellular Ca^{2+} accumulation on autoregulatory factors d_1 (fd_1), which in some bacteria are represented by alkylhydroxybenzene derivatives [2, 3] and exhibit membranotropic activity [4, 5] and properties of chemical chaperons [6] (according to Hecco's terminology [7]).

The effect of AHB is based on their capacity to form intermolecular hydrogen bonds and to participate in hydrophobic and electrostatic interactions; they change cell membrane viscosity [4, 5] and spatial structure of biomacromolecules, including enzymes [5, 6], which become more stable and change their functional activity [5–7].

It should be noted that stationary-phase and, especially, resting cells, in the control of whose development AHB is involved, exhibit increased resistance to unfavorable stressful impacts (temperature and pH shocks, UV radiation, etc). X-ray microanalysis revealed another feature of stationary-phase and resting cells of bacteria and yeasts: their content of Ca^{2+} is higher than that in proliferating cells [8].

The above properties of fd_1 explain why their effect on the stress response is not species-specific and is observed not only with microbial [9] but also with animal cells [10]. We considered it pertinent to determine

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the AHB influence on the intracellular pool of Ca^{2+} in mycelial fungi under stressful conditions. Regulation of the Ca^{2+} response in micromycetes has been poorly studied. In addition, these microorganisms are convenient test objects because the mutant strain *Aspergillus awamori* 66A contains the recombinant photoprotein aequorin, the photon emission of which depends on the intracellular (cytosol) Ca^{2+} concentration [11]. By using the recombinant aequorin method, it is possible to determine both the amount of free intracellular Ca^{2+} and the dynamics of the Ca^{2+} response.

In this work, the effect of a chemical analogue of fd_1, 4-*n*-hexylresorcinol, on the Ca^{2+} response of *Aspergillus awamori* cells was studied under stressful conditions.

MATERIALS AND METHODS

The strain *Aspergillus awamori* 66A was kindly provided by the Laboratory of Micromycete Cells, Edinburgh University. *A. awamori* is a mutant strain that produces the photoprotein aequorin [11]. The micromycete was grown at 30°C for 72 h in liquid Vogel medium [12] supplemented with 1% sucrose, using a 96-well plate. Into each well, 100 μl of a spore suspension with a spore concentration of 5×10^5 cells/ml was introduced.

Micromycetes were exposed to mechanical shock by agitation of the growing cultures on a shaker (120 rpm) for 5 min.

Experiments were performed with a chemically synthesized alkylhydroxybenzene, 4-*n*-hexylresorcinol (Serva). Using injectors installed into the luminometer, an AHB ethanol solution was introduced into each well. The final ethanol concentration was 0.016%.

The interaction of the photoprotein aequorin with Ca^{2+} results in aequorin cleavage to apoaequorin and the chromophore coelenteramide, which is accompanied by light emission. The intensity of luminescence is proportional to the Ca^{2+} concentration in the cytosol. Restoration of aequorin was brought about by the addition of 40 μl of a coelenterazine methanol solution to each well, to a final concentration of 2.5 μM. The coelenterazine solution was freshly prepared immediately before the addition to cell suspensions.

Light emission was measured using an EG&G Berthold LB96P Microlumat luminometer equipped with a photomultiplier; the luminometer was adapted to measurement of cell luminescence in the 96 wells of the plate.

The luminescence intensity, which correlated with the level of free cytosol Ca^{2+}, was expressed in relative light units. The Ca^{2+} concentration was calculated from the relative light units using the empirically derived formula

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

where k is the ratio of luminescence at a certain moment to the total maximum luminescence [13], defined as the value measured after total degradation of the cell aequorin caused by the addition of $CaCl_2$ to the cell suspension to a final concentration of 1.5 M. All experiments were performed in five replicates. The results were processed statistically using variation statistics analysis and the significance level $P < 0.05$.

RESULTS

We have previously shown that, in growing bacterial and yeast cultures, the level of cellular Ca^{2+} increases significantly upon an increase in the extracellular AHB caused by starvation stress or other unfavorable conditions [14–16]. Similar changes in cell ionic homeostasis were observed when exogenous AHB were introduced into microorganism suspensions [8]. Therefore, in the present work, we first studied the ability of AHB introduced at various concentrations (0.001 and 0.01%) to induce a Ca^{2+} response of cells of mycelial fungi. The level of response was determined from the mode and intensity of luminescence in 24- and 72-h *A. awamori* cultures (the exponential and stationary growth phases, respectively) (Figs. 1, 2). The control fungal cultures were exposed to mechanical treatment (agitation of the suspension), which was previously shown to increase the level of Ca^{2+} in cells [16]. In both control and experiment, the cell Ca^{2+} concentration increased abruptly 1 min after treatment (agitation or AHB introduction) (Figs. 1, 2). Hence, an increase in extracellular AHB served as a stress signal for the cells. The response, i.e., the amplitude of luminescence, which correlated with the amount of Ca^{2+} in the cytosol, depended on the physiological age of the cells and the concentration of exogenous AHB. The response of exponential-phase cells to 0.001% AHB was lower than that of the stationary-phase cells, but, in both cases, the amplitudes were the same as those recorded in response to mechanical treatment. In response to 0.01% AHB, the amplitudes of luminescence were similar in the exponential and stationary cultures. Figure 3 shows the levels of Ca^{2+} accumulation in cells of the control and experimental variants. This parameter was calculated by integration of the entire area of Ca^{2+} response, in which a significant proportion falls on the area of response relaxation (Figs. 1, 2). In all variants, Ca^{2+} accumulation in cells depended on the micromycete age and increased with increasing AHB content in the medium. Note that the response of the stationary culture (72 h) to 0.01% AHB was lower than that of the exponentially growing 24-h culture, although the opposite dependency was observed in cases of the addition of 0.001% AHB or mechanical treatment. These results can be explained by the fact that, in stationary-phase microbial cultures, the d_1 (AHB) concentration increases by an order of magnitude to reach its maximum in both cells and culture liquid [14, 15]. In addition, the content of saturated fatty acids in pro- and eukaryotic cell membranes and, hence, the microviscosity of the membranes increase [17]. Therefore, addi-

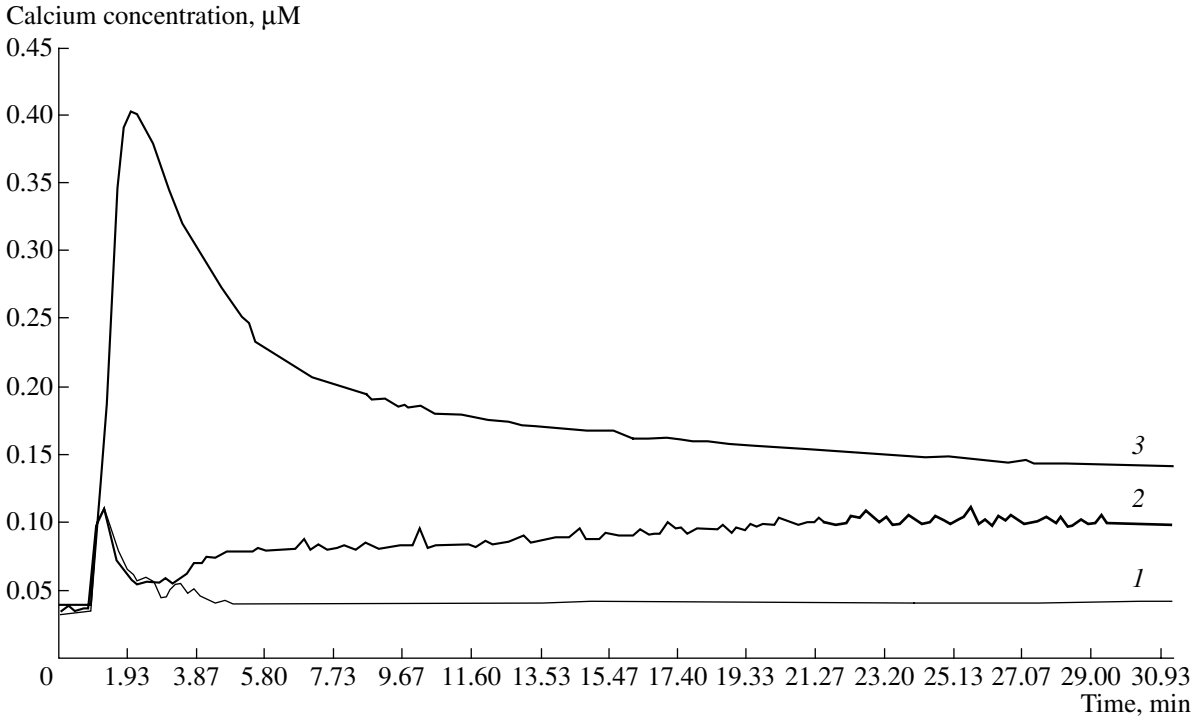

Fig. 1. Dynamics of Ca^{2+} ions in 24-h *A. awamori* cells grown (*1*) in the absence of AHB (control) and in the presence of (*2*) 0.001 and (*3*) 0.01% AHB.

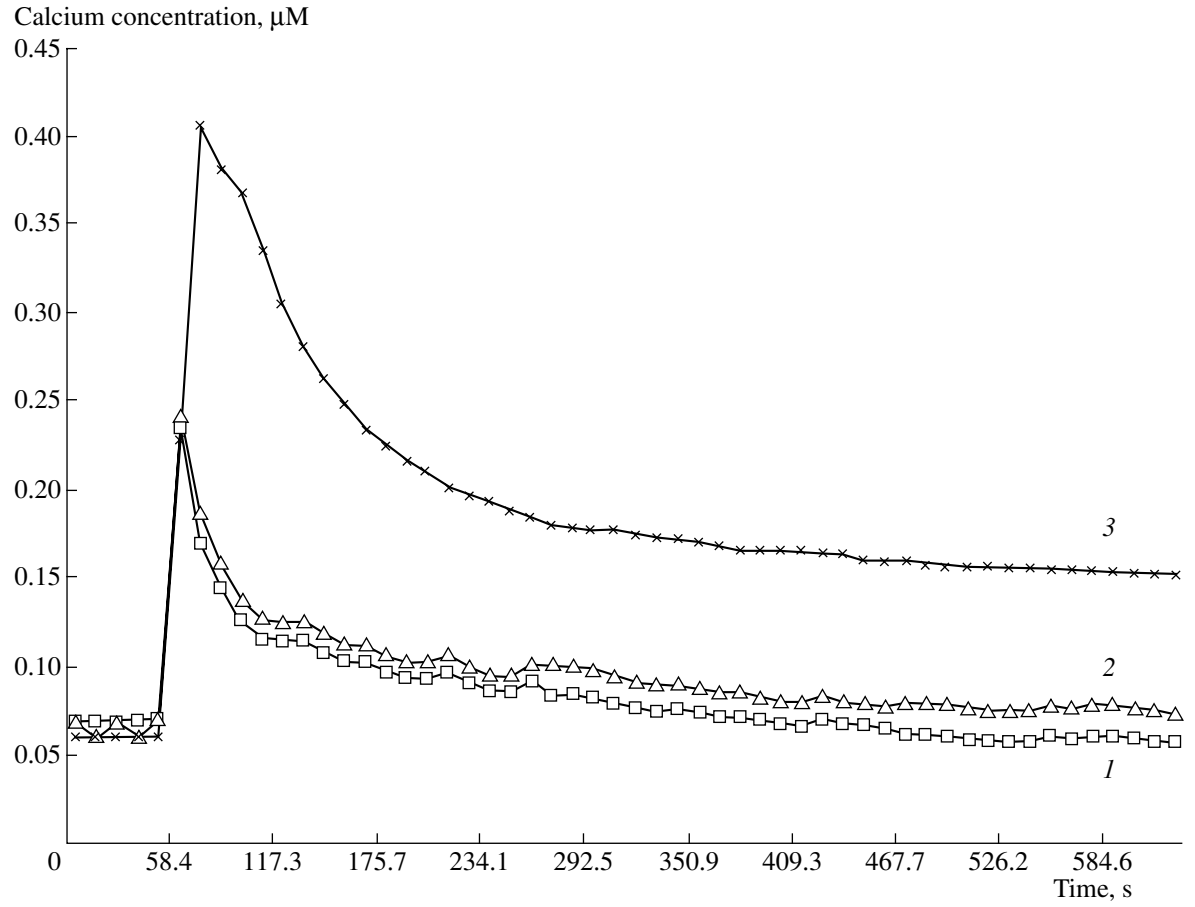

Fig. 2. Dynamics of Ca^{2+} ions in 72-h *A. awamori* cells grown (*1*) in the absence of AHB (control) and in the presence of (*2*) 0.001 and (*3*) 0.01% AHB.

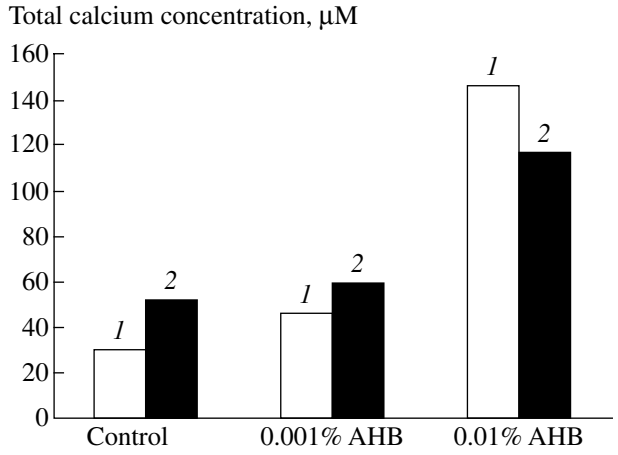

Fig. 3. AHB-stimulated accumulation of Ca^{2+} in *A. awamori* cells as dependent on the culture age: (*1*) 24-h culture; (*2*) 72-h culture.

tional introduction of 0.01% AHB results in their supraoptimal concentration, which inhibits membrane functional activity, including Ca^{2+} transport. The dose-dependent effect of AHB on the respiratory activity of membranes has been well documented [2, 3, 5, 15, 18].

It should be noted that 0.001% AHB stimulated a primary Ca^{2+} response in both 24- and 72-h cultures. The intensity of this response (the amplitude of luminescence) was higher than that of the response to mechanical impact (control) (Figs. 1, 2); however, AHB caused higher Ca^{2+} accumulation (Fig. 3). Similarly, the amplitudes of 24- and 72-h culture responses to 0.01% AHB were the same (Figs. 1, 2), whereas these cultures differed in Ca^{2+} accumulation (Fig. 3). A significant contribution of the secondary Ca^{2+} response, relaxation, to the Ca^{2+} accumulation accounts for these results. The secondary response, which developed within 4 min after the addition of various AHB concentrations, consisted in stabilization of the increased level of intracellular Ca^{2+}. This effect was most pronounced in 24-h cultures supplemented with 0.001% AHB (Fig. 1). Thus, the above distinctions in the Ca^{2+} accumulation in *A. awamori* cells can be explained by the secondary response (Fig. 3). The fact that AHB accounts for a high Ca^{2+} concentration maintained for a long time in micromycete cells suggests that AHB may protect cells from stressful impacts.

The protective effect of AHB on the *A. awamori* cells exposed to mechanical impact was evaluated from the cell Ca^{2+} response. In this series of experiments, the AHB concentration was 0.01%. First, this concentration caused a pronounced cell response, and, in addition, AHB solubility sharply decreases at higher doses, so that opalescence of the solution was observed (note that the final concentration of ethanol was 0.016%). Ten-minute preincubation of 24- and 72-h micromycete cells in the presence of 0.01% AHB increased the level of Ca^{2+} in the cytosol to 0.14–0.15 μM. Against the background of this Ca^{2+} content, subsequent mechanical stimulation induced a lower Ca^{2+} response than in the control (cells exposed only to mechanical stimulation) (Figs. 4, 5). The increased content of Ca^{2+} in the cytoplasm of the experimental cells was maintained for a long time,

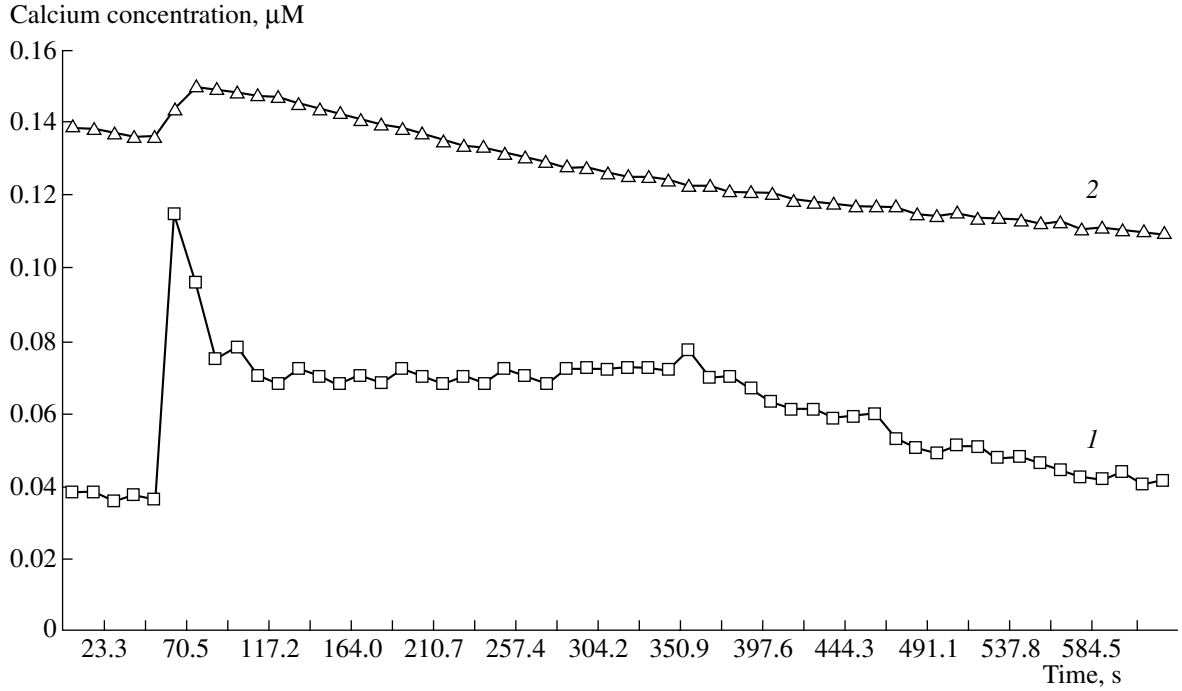

Fig. 4. Dynamics of Ca^{2+} in the cytosol of 24-h *A. awamori* cells exposed to mechanical stress after 10-min preincubation with AHB: (*1*) cells exposed to the mechanical stimulus only (control); (*2*) cells preincubated with 0.01% AHB prior to the mechanical stimulation.

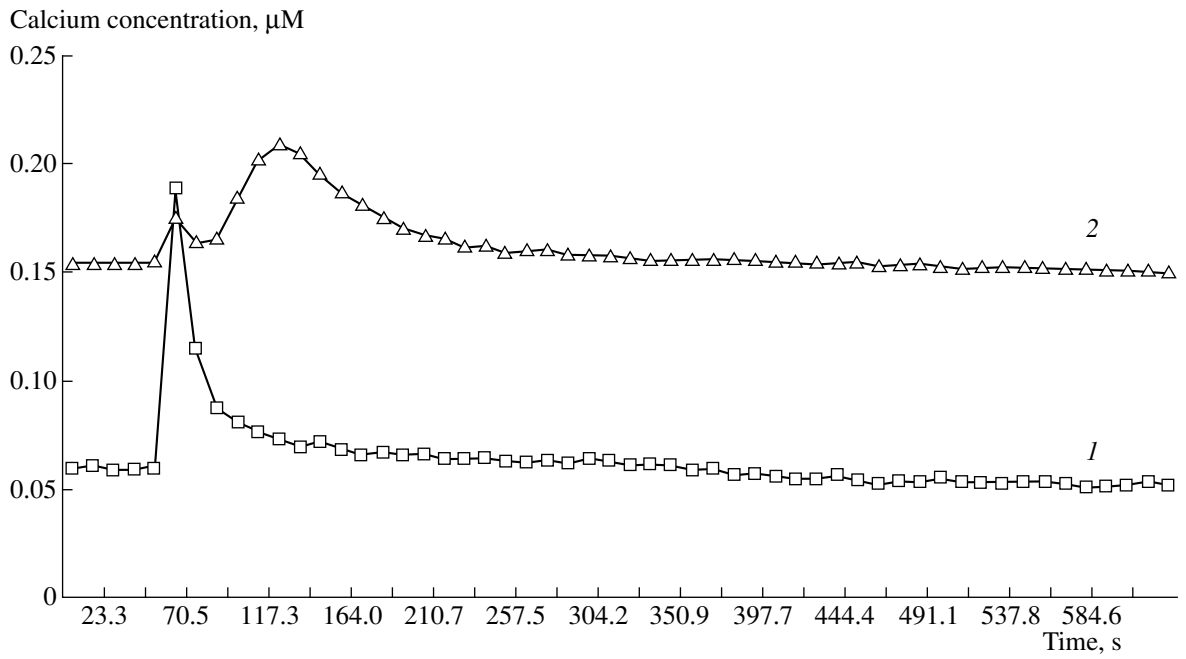

Fig. 5. Dynamics of Ca^{2+} in the cytosol of 72-h *A. awamori* cells exposed to mechanical stress after 10-min preincubation with AHB: (*1*) cells exposed to the mechanical stimulus only (control); (*2*) cells preincubated with 0.01% AHB prior to the mechanical stimulation.

which was not characteristic of the control cells. The secondary increase in the amplitude of luminescence and, thus, in the Ca^{2+} concentration was most pronounced in cells of 72-h cultures (Fig. 5). In the experiment, the integral Ca^{2+} concentration was 2.5-fold higher than in the control (Fig. 6).

The significant changes induced by cell preincubation with AHB in the parameters of response to mechanical impact demonstrate a considerable increase in cell stability. In these AHB-protected cells, the Ca^{2+} response to mechanical impact was lower than in the control cells, but the very fact of the cell capacity for response and subsequent relaxation testifies to normal functioning of the calcium channels in AHB-treated cells.

The protective effect of AHB was also confirmed in experiments with an inverted sequence of treatments. The cells were first exposed to mechanical impact, and an AHB solution (0.01%) was added after cell reversion to the initial state (as judged from the Ca^{2+} response), 15 and 60 min after mechanical impact. In a 24-h culture, the amplitude of cell response to AHB was more pronounced 60 min after mechanical stimulation (Fig. 7), whereas, in a 72-h culture, the amplitudes were similar after 15 and 60 min (Fig. 8). In both 24- and 72-h cultures, a high level of Ca^{2+} was retained in the cytosol after AHB addition and it failed to decrease to the initial Ca^{2+} content after mechanical stimulation (Figs. 8, 9). Thus, the mechanical stimulation induced a short-term Ca^{2+} response, but the cells remained unprotected from the following stressful impacts, particularly, from that of AHB introduction.

DISCUSSION

By the example of 4-*n*-hexylresorcinol, we have shown in this study that alkylhydroxybenzenes, the autoregulators of microbial anabiosis, protect cells of mycelial fungi from adverse impacts. It should be noted that AHB (d_1 factors) produce a nonspecific effect on various biological objects because they interact with cell components (proteins and lipids) via hydrogen bonds and hydrophobic and electrostatic binding. Thus,

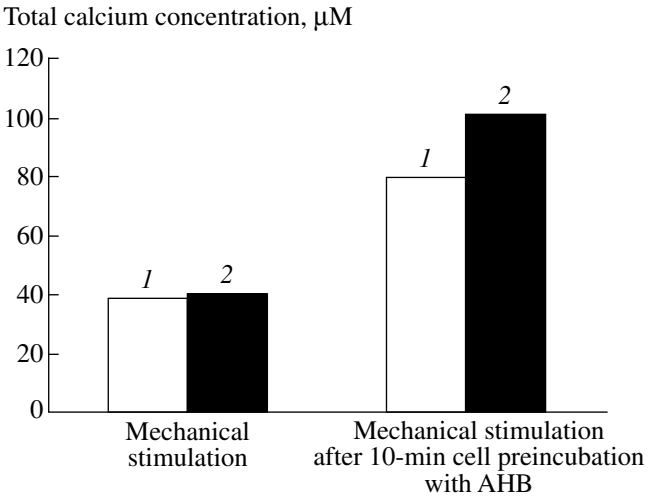

Fig. 6. Effect of 10-min cell preincubation with 0.01% AHB on Ca^{2+} accumulation in the cytosol of *A. awamori* cells of different age exposed to mechanical stress: (*1*) 24-h culture; (*2*) 72-h culture.

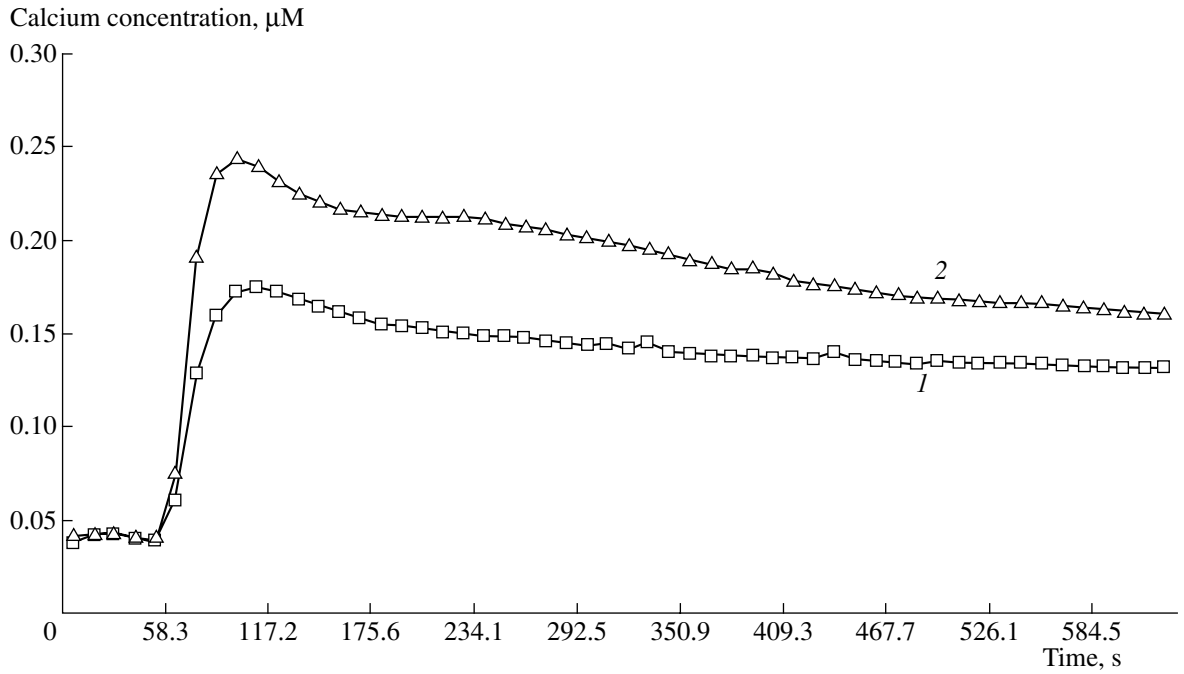

Fig. 7. Ca^{2+} response of 24-h *A. awamori* cells to a mechanical stimulus followed by the addition of 0.01% AHB: (*1*) AHB added after 15 min; (*2*) AHB added after 60 min.

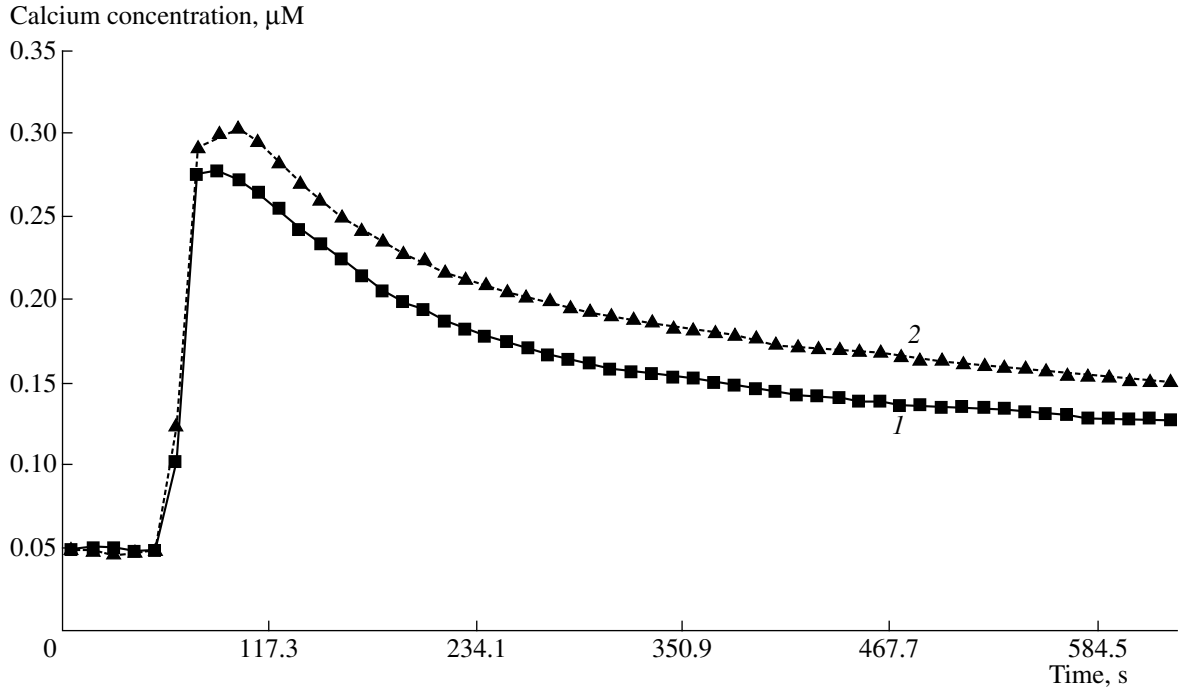

Fig. 8. Ca^{2+} response of 72-h *A. awamori* cells to a mechanical stimulus followed by the addition of 0.01% AHB: (*1*) AHB added after 15 min; (*2*) AHB added after 60 min.

we have shown previously that 4-*n*-hexylresorcinol protects ras-transformed mouse macrophages from gene-toxic concentrations of RNase and from the chemical agent ethanol (5 vol %) [10]. Therefore, the protective effect of AHB on lower eukaryotes, mycelial fungi, was an expected phenomenon. In this study, we have shown for the first time that, along with other mechanisms, the regulatory influence of AHB on Ca^{2+} accumulation in cells accounts for the protective effect of AHB. Ca^{2+} is known to trigger a cascade of cell reac-

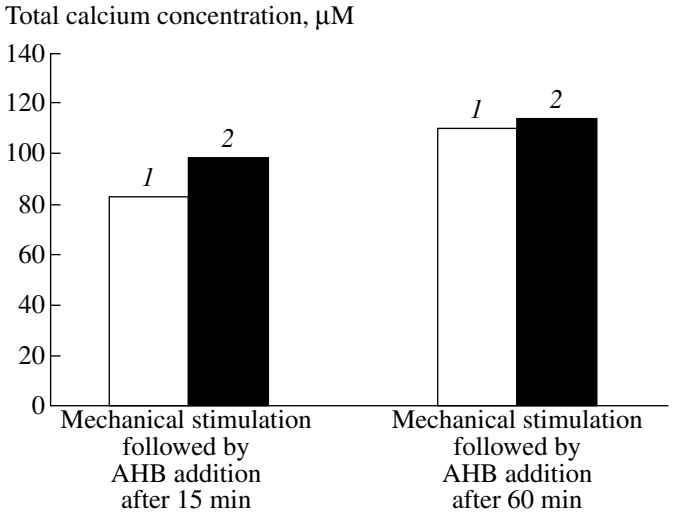

Fig. 9. Ca^{2+} accumulation in *A. awamori* cells 1 h after cell exposure to a 5-min mechanical stimulus followed by the addition of 0.01% AHB as dependent on the culture age: (*1*) 24 and (*2*) 72-h cultures.

tions during the stress response [19]. An increase in the level of AHB in *A. awamori* culture led to a pronounced increase in the content of free Ca^{2+} in the fungal cell cytoplasm, which was retained for a long time. Thus, 3–4 min after the mechanical treatment of cells, the Ca^{2+} concentration decreased to the initial level, whereas, after AHB addition, the high level of Ca^{2+} was retained for the entire time of the experiment. These results suggest that AHB influence the mechanisms that underlie both Ca^{2+} accumulation and Ca^{2+} export from the cytoplasm (Ca^{2+} ATPases and Ca^{2+}–H^+ antiport). Given that the Ca^{2+} ATPase of the plasma membrane responds to an even insignificant increase in the cell Ca^{2+} pool, it may be assumed that AHB modifies the Ca^{2+} ATPase protein. Earlier, we showed the ability of AHBs to modify such enzymes as trypsin, chymotrypsin, glucose oxidase, invertase, and RNase [6, 20].

AHB may also play a regulatory role in the stabilization and modulation of the activity of polysubunit enzymes and complex protein assemblages. Thus, some alkylresorcinol homologues were shown to control melanophore aggregation in amphibian pigment cells—a process that controls a system of tubulin proteins. Certain AHB concentrations caused melanophore aggregation irrespective of changes in the light regime and the sequence of treatment with AHB solutions (unpublished data). To a certain degree, this is similar to the effect of stabilization of the increased Ca^{2+} level in AHB-treated fungal cells.

The secondary Ca^{2+} response of the aspergillic cells was another interesting phenomenon well-detectable after the addition of low AHB concentrations during exponential growth (Fig. 1) and in the case of mechanical impact on AHB-preincubated stationary-phase cultures (Fig. 5). This effect was the most pronounced in stationary-phase (72-h) cultures, where the viscosity of the cell membranes was higher than in exponentially growing cultures and the activity of the membranes was changed. The very fact of the secondary Ca^{2+} response may testify to repeated stimulation of Ca^{2+} channels. Changes both in the conformation and activity of the channel proteins and in the lipid–protein interactions within altered membranes may account for this effect. AHB were found to perform functions of chemical chaperons; they control conformational changes in proteins and modulate protein activity [6, 20]. The modifying effect of AHB on the membrane lipid stroma is a less likely explanation for the secondary Ca^{2+} response, although the membranotropic activity of AHB has been reported in a number of papers [4, 5, 18]. The effect of 4-*n*-hexylresorcinol on membrane lipids is opposite to that discussed above: an increase in AHB concentration leads to polycrystallization of the membrane lipid stroma [4, 5]. However, the cell stress response to increasing AHB concentration may include intensification of lipid synthesis to compensate for the decrease in the membrane fluidity directly caused by AHB. As

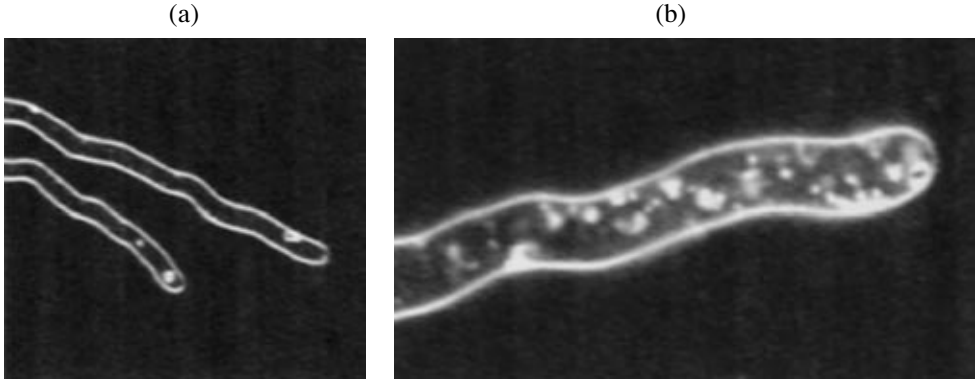

Fig. 10. Confocal microscopy of *A. awamori* cells (FM464 dye) incubated with 0.01% AHB for (a) 1 and (b) 2 h.

shown in preliminary experiments using confocal microscopy and lipid staining with FM464 dye, the lipid component did accumulate during incubation of *A. awamori* cells with AHB (Fig. 10). At a 0.01% AHB concentration, the membrane structures and some intracellular organelles were enriched with lipids. The reaction intensity increased with incubation time throughout the entire cell. These preliminary results are of interest for further studies of the physiological mechanisms that underlie cell response to stressful impacts and of the involvement of the autoregulatory d_1 factors (AHB) in these mechanisms.

Thus, microconcentrations of extracellular nonspecific microbial inducers of anabiosis, which are represented by alkylhydroxybenzenes (AHB) in a number of microorganisms and are not species-specific, cause changes in the Ca^{2+} response of *A. awamori* cells. The AHB effect depends on the concentration in the culture and on the physiological age of the cells. AHB serve as natural protectors that enhance intracytoplasmic Ca^{2+} content and maintain its increased level, contributing to the protection of cells from stressful impacts.

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