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EXPERIMENTAL ARTICLES

The Relationship between Cellular and Calcium Responses of *Aspergillus awamori* to External Influences

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Abstract—The cellular and Ca^{2+} responses to physiological stimuli of different nature were studied in the experiments with the strain *Aspergillus awamori* 66A containing recombinant aequorin, a Ca^{2+}-dependent photosensitive protein. The relationship between the cellular response registered by changes in the development of the mycelial fungus (colony growth, hyphal branching, and the rate of spore formation) and the level and duration of calcium flares in the cytosol was assessed. The physical or chemical stimuli (mechanical effect, osmotic shock) inducing short-time calcium flares in the cytosol did not influence significantly the development of *A. awamori* grown in liquid or on solid nutrient media. The action on the 24-h *A. awamori* culture of the Ca^{2+}-selective ionophore (A23187) inducing long-term changes in calcium homeostasis caused disorders in the fungus development and morphology (hyperbranching of mycelial hyphae, formation of spherical cells, and inhibition of colony growth and spore formation). Thus, it was established that the development of cellular response in the micromycete correlated with the duration of the calcium flare in the cytosol.

Key words: *Aspergillus awamori*, recombinant aequorin, stress influences, Ca^{2+} dynamics, cellular response.

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Calcium is involved in transduction of diverse external stimuli and hormonal signals as a universal component of the signal systems in mammalian and plant cells [1, 2]. The functions of Ca^{2+} as a messenger are confirmed by the fact that, in response to any environmental change, a primary increase in the cytosol concentration of calcium occurs, which is a characteristic indicator of the stress state of the cells [3]. A sufficient range of methodical developments exists concerning the control mechanisms for the Ca^{2+} level in eukaryotes, making it possible to reveal the regulatory role of intracellular calcium in many physiological processes, including cell growth and division and the formation of microbial resting forms. However, few reports are known concerning the role of Ca^{2+} in the regulation of the apical growth of hyphae and mycelial branching in micromycetes [4, 5]. The absence of reproducible and reliable methods of monitoring the intracellular Ca^{2+} level in intact cells deterred the studies of the role of Ca^{2+} in micromycetes. When the micromycete strains were obtained with the gene encoding the photoprotein aequorin introduced by transformation, investigators had an opportunity to record the changes in Ca^{2+} level in the fungus cytosol in response to versatile external influences [6].

Earlier, using strain *Aspergillus awamori* 66A with the expressible gene of the protein aequorin, we showed that different physiological stimuli induced an increase in the Ca^{2+} level in the cytosol of micromycete cells differing in time. Each type of influence was characterized by a specific Ca^{2+} response, observed as differences in the time of increase of the cytosol Ca^{2+} concentration, the amplitude of the increase, and the duration of retention of the high calcium level. Using the specific Ca^{2+} transport inhibitors and agonists, it was shown that both the environment and intracellular Ca^{2+} reservoirs were the source of Ca^{2+} arriving in the cytosol on exposure to the studied physiological stimuli [7].

The aim of this work was to reveal the interrelationship between changes in the level of intracellular Ca^{2+} caused by the influence of different physiological stimuli on the micromycete and the growth and development of the fungus *A. awamori* 66A.

MATERIALS AND METHODS

The mutant strain *Aspergillus awamori* 66A with an expressible gene of the photoprotein aequorin obtained from the Edinburgh University Laboratory for Studying Micromycete Cells was the subject of the study [6].

A. awamori 66A was cultivated at 30°C in Vogel liquid medium with the addition of sucrose (medium VS)

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and on a solid medium (2% of agar) of the same composition enriched with glucose (medium GEC) [8].

The spore (conidium) suspension in 0.5 ml of 0.8% NaCl (PZ) was used as inoculation material. The spore biomass was obtained by washing the conidia off the surface culture with the buffer solution (PZ). The spores were washed off the medium and the mycelium remnants and, after centrifugation (5000 *g*), resuspended in PZ and stored at 4°C. The spore concentration was standardized in the hemocytometer after tenfold dilution; their number was 500000 spores/ml.

The measurement of aequorin luminescence was carried out in the cells of *awamori* 66A culture grown on a liquid or solid medium in the 96-well plate. The following components were introduced into each well: (a) 100 μl of agarized medium and (b) 100 μl of liquid Vogel medium. After filling the wells with medium, 20 μl of Vogel medium containing the spore inoculum (the end concentration varied from 500 to 500000 spores/ml) and selentrasin was introduced into each well. The culture incubation conditions were the same as those described in [7].

The micromycetes were exposed to mechanical influences, osmotic shock, and high calcium concentrations as described earlier [7]. The effect of these external stimuli on the fungus development was recorded by the number of branches formed on one germ tube from each spore of *A. awamori* 66A. For this purpose, the spores in Vogel liquid medium (the suspension density varied from 500 to 500000 spores/ml) were incubated for 24 h at 30°C on a coverslip placed in a moist chamber to avoid desiccation. In the case of each type of influence, 75 microcolonies (25 per one coverslip) were analyzed. They were counted every 2 h for 1–7 h after the 24-h cultures had been subjected to the relevant stimulation. Intact cells served as the control.

In order to determine the effect of A23187 on branching of the mycelial hyphae of *A. awamori* 66A, a method similar to that described above was used in Vogel liquid medium. A23187 (50 μl) (at final concentrations of 10 and 50 μM) was added to every drop (50 μl) of the 24-h culture, and the rate of hyphal branching was determined after 1, 3, 5, and 7 h. The cells subjected to mechanical treatment (shaking) were used as the control.

The influence of A23187 on the expansion of the mycelial hyphae of *A. awamori* exposed to A23187 was studied when the fungus was grown from spores on sterile cellophane (8.5 cm in diameter) placed on the surface of agarized Vogel medium in a petri dish. The grown 24-h culture on cellophane was transferred to solid medium supplemented with the ionophore A23187. Vogel medium without the Ca^{2+}-ionophore was used as the control. The fungal biomass was sampled every 24 h for 8 days by excising it with a scalpel together with cellophane, and the samples were studied using a dark-field microscope. The branching expansion rate of *A. awamori* was assessed by the colony size (the radius, mm). The experiment was carried out three times with six dishes per each experimental variant.

The influence of A23187 on the morphology of the mycelial hyphae of *A. awamori* was determined after spore germination on solid Vogel medium (24 h, 30°C). For analysis, pieces of agar with mycelium (1 × 0.8 cm) were excised and placed upside down on a medium containing the preparation of A23187. No ionophore was introduced into the control samples. After 30 min and, subsequently, every hour, one piece of agar was added to a drop of the dye FM4-64 (Molecular Probes Inc., Oregon, United States) and placed on the microscope slide and the image of the hyphae was obtained by confocal laser scanning microscopy.

The micromycete viability was assessed by fluorescence microscopy of the cells stained with propidium iodide and fluorescein diacetate (FDA) [9]: the cells stained red with propidium iodide were considered dead; those stained green with FDA were considered living. The 50% level of dead cells was considered as the micromycete death 3 h after exposure to A23187.

The statistical data analysis was carried out with the Microsoft Excel 2000 software package. The standard deviation and the minimal significant deviation at a 5% level of significance were calculated.

RESULTS AND DISCUSSION

The morphological and physiological changes (colony growth rate, morphology of the apices of mycelial hyphae, and their branching) in the culture of *A. awamori* in response to external influences inducing a temporary increase in the Ca^{2+} content in the micromycete cytosol were studied [7]. In order to characterize the growth of the fungus in the control variants, the spores were inoculated into a drop of liquid medium (50 μl) on the coverslip, which was placed in the moist chamber to avoid desiccation. Of the inoculum concentrations checked (5×10^2–5×10^5 spores/ml), the optimum one was 5×10^4 spores/ml. In this case, the appearance of a germ tube with one or two branches was observed microscopically in the spores 4 h after incubation. Higher inoculum concentrations resulted in spore agglomeration and formation of a complex network of intertwined hyphae, which hampered the determination of the rate of their branching in the process of growth.

The morphology of the fungus growing in liquid medium was studied using confocal microscopy after exposure of the 24-h culture to the external stimuli inducing a short-term increase in the level of Ca^{2+} in the cytosol [7]: mechanical treatment, hypoosmotic shock, and an increase in the level of exogenous calcium by addition of 5 mM $CaCl_2$ to the medium. In all the exposure variants, as in the control, we observed increased hyphal branching with time, although, in

Table 1. Dynamics of hyphal branching from the germ tube after treatment with physicochemical stimuli (the number of hyphae)

Time after stimulation, h	Control	Mechanical treatment	Hypoosmotic shock	Addition of 5 mM $CaCl_2$
1	1.13 ± 0.05	1.16 ± 0.05	1.16 ± 0.05	1.16 ± 0.05
3	1.46 ± 0.08	1.44 ± 0.07	1.37 ± 0.08	1.47 ± 0.16
5	1.59 ± 0.10	1.59 ± 0.08	1.49 ± 0.14	1.48 ± 0.08
7	1.96 ± 0.12	1.92 ± 0.14	1.72 ± 0.9	1.6 ± 0.08

Note: The number of branches in the microcolonies of the 24-h culture grown in a drop of liquid medium was determined by confocal microscopy.

the latter two experimental variants, it was slightly less pronounced (Table 1).

Quite a different picture was observed when *A. awamori* was exposed to the Ca^{2+}-selective ionophore calcimycin A23187. This agonist is known to induce a release of Ca^{2+} into the cytosol from the intracellular calcium reservoirs (mitochondria) in *Penicillium notatum* [10], to aid in increasing the rate of hyphal branching in *Neurospora crassa* [11], and to inhibit budding in *Ophiostoma ulmi* [12]. Earlier, we showed the ability of calcimycin A23187 to increase the Ca^{2+} level in the cytosol of *A. awamori* cells grown in liquid medium [7]. In our experiments, in order to study the effect of A23187 on the development of the micromycete, it was also cultivated in liquid Vogel medium for 24 h with the subsequent addition of A23187 (to the final concentration of 10 and 50 μM). It was noted that calcimycin in aqueous solution forms micelles localized around the mycelial hyphae; the local concentration of the preparation is therefore likely to increase. Apparently, this explains the inhibition of branching of the micromycete hyphae in the experimental variants compared to the control during the whole observation period (7 h) after introducing A23187 into the culture (Table 2).

In order to obtain objective information about the effect of A23187 on the morphological and physiological changes in the micromycete, the fungus cultures in the subsequent experiments were grown in 96-well plates both in liquid and on agarized media. At first, the Ca^{2+} response of the 24-h culture cells to external stimuli was determined. It was found that, in both the submerged and surface growth of the micromycete, the lowest amplitude of the calcium response was recorded when the cells were subjected to mechanical treatment; the highest, when $CaCl_2$ (5 mM) was introduced (Fig. 1). In all the exposure variants, Ca^{2+} level in the cytosol quickly returned to the normal value, secondary calcium flares were not observed, and the rise and the lag period times were similar (Figs. 1a–1c). These results demonstrate the similarity of Ca^{2+} response to external stimuli irrespective of the medium in which the micromycete was cultivated.

Table 2. Dynamics of hyphal branching from the germ tube after exposure to the Ca^{2+} selective ionophore A23187 (the number of hyphae)

Incubation time, h	Control	Addition of 10 μM of A23187	Addition of 50 μM of A23187
1	1.27 ± 0.05	1.08 ± 0.05	1.06 ± 0.05
3	1.45 ± 0.09	1.09 ± 0.03	1.09 ± 0.03
5	1.50 ± 0.07	1.09 ± 0.03	1.09 ± 0.03
7	1.90 ± 0.12	1.09 ± 0.03	1.09 ± 0.03

Note: The number of branches in the microcolonies of the 24-h culture grown in a drop of liquid medium was determined by confocal microscopy. The cells subjected to mechanical treatment served as the control.

The next stage of the work involved investigation of the effect of calcimycin A23187 on formation of the Ca^{2+} response of the micromycete cells growing in liquid and solid media in the wells. The preparation of A23187 (10 μM) was introduced into 24-h cultures of *A. awamori*. The micromycete cultures subjected to mechanical treatment served as the control. Ca^{2+} response in the experimental and control variants was recorded for 10 min and 5 h after the stimulation. The measurements of the Ca^{2+} dynamics for 10 min showed that, in both cases (shaking or the introduction of A23187), a jumplike increase in the Ca^{2+} concentration in the cells occurred 1 min after the treatment. The character of Ca^{2+} response did not differ in the cells growing in liquid or on solid media, but the luminescence amplitude for the cells in submerged culture was lower (Fig. 2a). The measurements taken for 5 h after exposure revealed substantial differences in the Ca^{2+} response of the cells to the introduction of A23187 and shaking. Thus, when A23187 was introduced, the mycelium growing both in a liquid and solid media exhibited a secondary flare of luminescence with the same amplitude and duration for both media (Fig. 2b).

Thus, the calcium response of *A. awamori* to the effect of the agonist A23187 is of a biphasic character, which reflects the entrance of Ca^{2+} into the cytosol from the environment and cellular reservoirs. Certain agonists are known to inhibit the activity of Ca^{2+}-ATPases and, thus, to prevent Ca^{2+} afflux into the cellular organelles, which ensures a high Ca^{2+} level in the

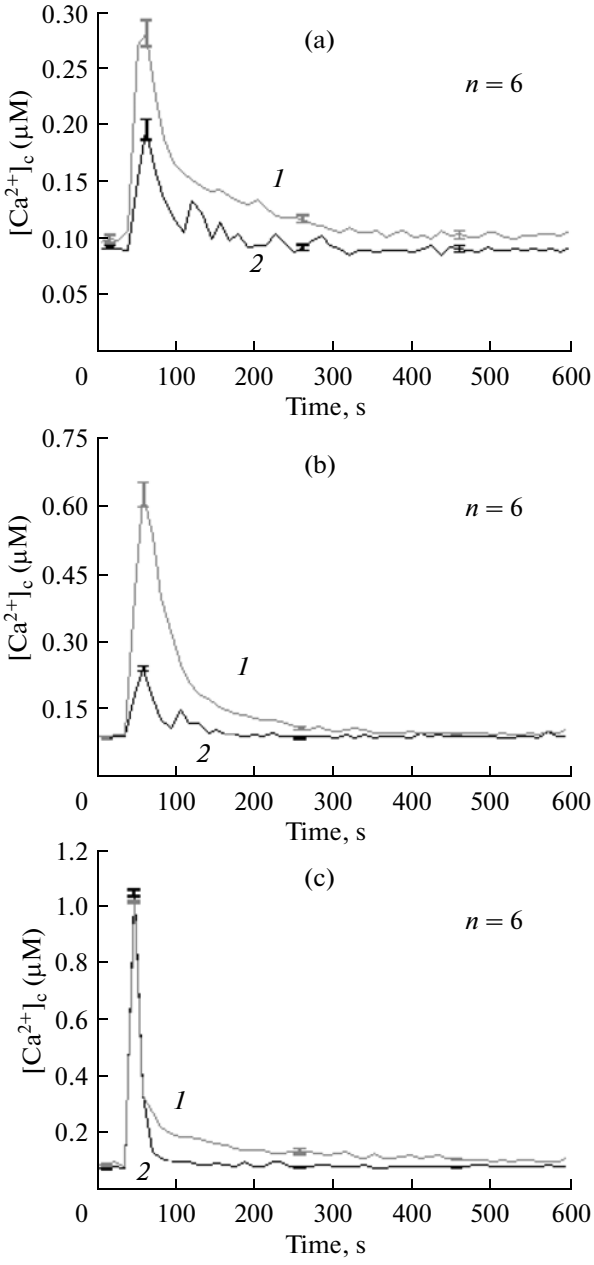

Fig. 1. Influence of mechanical shaking (a), hypoosmotic shock (b), and extracellular $CaCl_2$ (5 mM) (c) on the Ca^{2+} content in the cells of the cultures grown on solid (*1*) and in liquid media (*2*). The results represent the mean of six measurements ($n = 6$) the standard deviation with the use of the protocol of repeated measurements. The time of one cycle was 11.6 s.

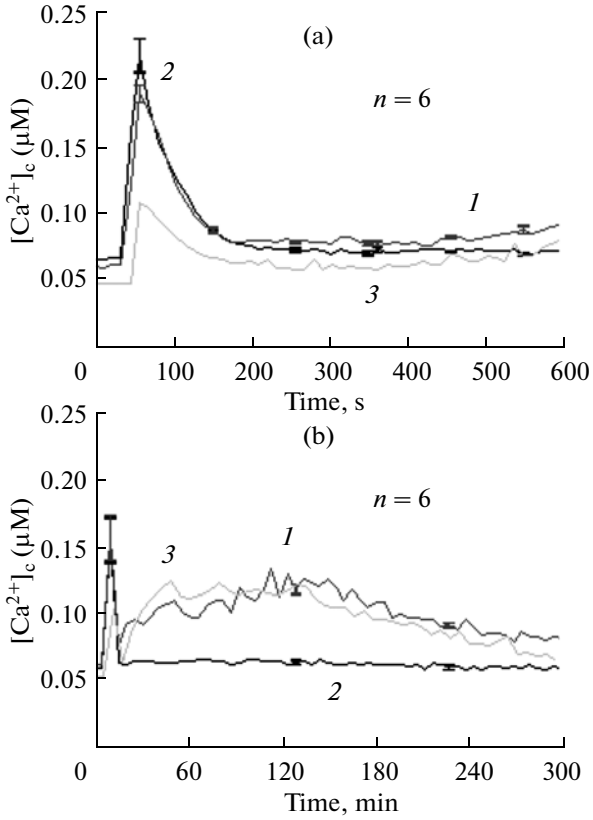

Fig. 2. Effect of A23187 on the Ca^{2+} content in the cells of the cultures grown on solid and in liquid media for 10 min (a) and 5 h (b) after the treatment. The media: solid (*1*), liquid (*3*), and the control (solid medium without A23187) (*2*). The experiments were carried out with the use of the protocol of repeated measurements ($n = 6$). In the experiment (a), the time of one cycle was 11.6 s; in the experiment (b), the time of one cycle was 5.2 min.

cytosol [12]. Analysis of these data suggests the presence of a special mechanism for the regulation of Ca^{2+} transport to/from the *A. awamori* cellular organelles into the cytosol. According to the data of [13], the secondary flare and long-term maintenance of the cytosol level of calcium on exposure of the micromycete to calcimycin A23187 probably result from its capacity for forming stable complexes with bivalent cations. Note that high Ca^{2+} concentrations are toxic to a cell due to its interaction with phosphate-containing compounds [14]. In our experiments, the normal level of Ca^{2+} concentration in the micromycete cells was not restored for a long time (Fig. 2b), which possibly had a negative effect on the development and viability of *A. awamori*.

Therefore, the next task was to determine the correlation between the A23187-induced changes in the Ca^{2+} balance in the cytosol and the growth of the *A. awamori* culture. Application of confocal microscopy revealed a number of methodical advantages of the analysis of growth of *A. awamori* on solid medium, rather than in submerged culture: the hyphae could be placed in the focus of one visual plane, the molecules of the agonist added to solid medium did not aggregate around the mycelial hyphae, and it was possible to use objectives with a higher magnification (40 and 60 times) and a short working distance. The study of the cellular response of the mycelial fungus to the presence of A23187 (10 μM) revealed significant changes in the morphology and hyphal branching of

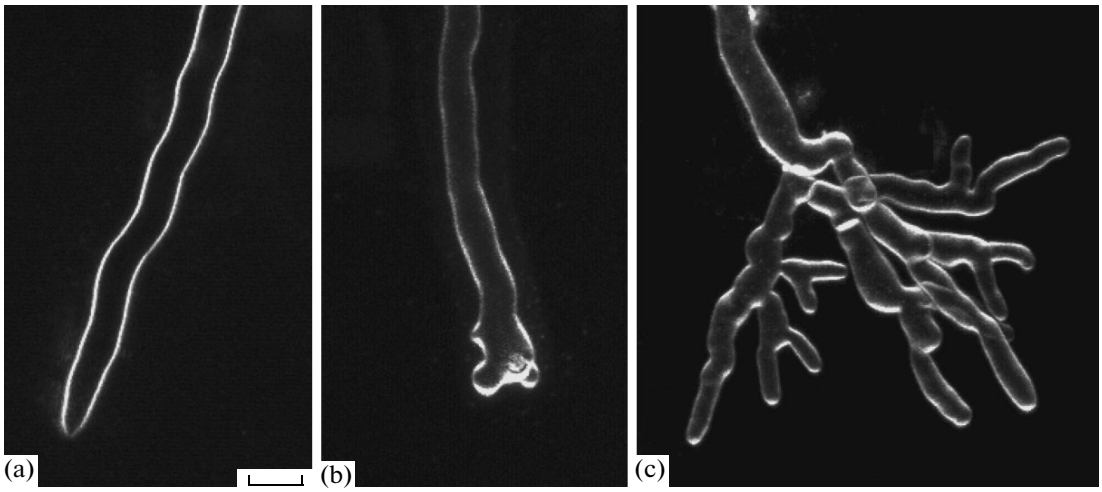

Fig. 3. Effect of A23187 on the morphology and branching of the mycelial hyphae of *A. awamori* grown on solid Vogel medium for 24 h: immediately after the introduction of A23187 into the 24-h culture (control) (a) and after 3 (b) and 5 h (c). The confocal images were obtained on addition of the dye FM4-64 with a 60× oil objective. Scale bar: 10 μm.

the micromycete cultivated on solid Vogel medium. After 3 h of incubation with A23187 (Fig. 3b), the hyphal tips became swollen compared to the control (Fig. 3a); bubbles were formed, which ruptured with time, possibly causing the death of the hyphae. Similar morphological changes in the fungi exposed to calcimycin were noted earlier for the cultures of *N. crassa* and *Fusarium graminearum* [15, 16]. Hyperbranching of the hyphae and sometimes the formation of spherical cells were the results of long-term Ca^{2+} response to A23187 (Fig. 3c).

These morphological changes were accompanied by a considerable decrease in the colony expansion rate (Table 3). As seen from these data, deceleration of the colony growth rate of *A. awamori* occurred immediately after the cultures were introduced on the A23187-containing medium: after 48 h, the colony radius was 55% of the control; and by the end of the experiment (after 192 h), only 38% compared to the control.

The findings revealing substantial changes in the fungus morphology and growth processes in the presence of A23187 suggest that the Ca^{2+} imbalance induced by A23187 in the cells exerted a toxic effect. This was experimentally confirmed by counting the number of dead and live cells in the experimental and control culture samples taken from the wells with agarized medium. Fluorescence microscopy of the cells stained with a mixture of dyes (propidium iodide and fluorescein diacetate) was carried out in ten microscope fields, and the live/dead cell ratio was determined. After 3-h incubation with A23187 (10 μM), about 50% of the hyphae were killed in the experimental samples, while in the control samples, only 5 and 95% of the hyphae survived.

Thus, the agonist A23187, causing long-lasting Ca^{2+} flares in the cytosol, at the concentration used

Table 3. Effect of the Ca^{2+}-selective ionophore A23187 on the growth rate of *A. awamori* colonies (radius, mm) on solid medium

Time after stimulation, h	Control	Treatment with A23187 (10 μM)	% of colony growth inhibition (in relation to the control)
24	1.0 ± 0.05	1.0 ± 0.05	100
48	2.9 ± 0.08	1.6 ± 0.07	55
72	6.0 ± 0.10	2.6 ± 0.08	43
96	8.5 ± 0.12	3.5 ± 0.09	41
120	15.0 ± 0.11	6.0 ± 0.10	40
144	19.0 ± 0.15	7.6 ± 0.12	40
168	25.5 ± 0.17	9.7 ± 0.15	38
192	30.4 ± 0.19	11.6 ± 0.14	38

(10 μM) prevents to a significant degree the normal growth and branching of mycelial hyphae, which leads to partial death of *A. awamori* cells. This agrees with the literature data on the influence of Ca^{2+} on such processes as the cell cycle in *Aspergillus nidulans* [17], hyperbranching in *A. fumigatus* [18], and hyphal growth and branching in *Botrytis cinerea* [19]. However, the link between the duration of disrupted Ca^{2+} balance in a cell under stress and the cellular response has not been known so far. The data obtained confirmed that the obligatory condition of the preservation of cell viability and of the functioning of the calcium signal system is a decrease in the cytosol calcium ion level to the initial one [20] following the calcium flare, because the disruption of Ca^{2+} homeostasis induced by A23187 was the main cause of inhibition of growth of the *A. awamori* culture and its death. The link between the duration of Ca^{2+} response of the cells and their death revealed in micromycetes does not run counter to the literature data according to which A23187-induced disruption of calcium homeostasis triggers cell apoptosis [21].

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