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Effect of Amiodarone on Thermotolerance and Hsp104p Synthesis in the Yeast *Saccharomyces cerevisiae*

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Abstract—Amiodarone (AMD) is known to induce a transient increase in cytosolic Ca^{2+} level in cells of the yeast *Saccharomyces cerevisiae*. In the present study the effect of AMD on the thermotolerance and Hsp104p synthesis of the yeast was studied. AMD induced Hsp104p synthesis and increased survival of the yeast after a severe heat shock (50°C). The development of thermotolerance to a considerable extent depended on the presence of Hsp104p. The same effect was achieved by treatment with the classical uncoupler CCCP, which is also known to increase the cytosolic Ca^{2+} level. It is supposed that the change in intracellular Ca^{2+} concentration plays an important role in activation of the *HSP104* gene expression and in increasing the thermotolerance of the yeast. The possible link between mitochondrial activity and calcium homeostasis is discussed.

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Key words: amiodarone, *Saccharomyces cerevisiae*, thermotolerance, Hsp104p, mitochondrion

In all studied species, mild heat shock itself does not cause damage, but induces heat shock protein (Hsp) synthesis followed by development of resistance to subsequent more severe heat shock. This phenomenon is called acquired (induced) thermotolerance [1]. Many Hsp act as molecular chaperones to repair cell proteins damaged by heat [2]. In *Saccharomyces cerevisiae* the protein Hsp104p, a member of Hsp100/ClpB (caseinolytic protease B) family, is shown to play the crucial role in acquired thermotolerance [3]. Under the heat shock conditions the *HSP104* gene expression is regulated by heat shock transcription factors Hsf1p and Msn2/Msn4p (multicopy suppressor of *SNF1* mutation), which bind with regulatory *HSE* (heat shock element) and *STRE* (stress responsive element), respectively, localized in its promotor [3].

Abbreviations: AMD, amiodarone (2-butyl-3-(3,5-diiodo-4-diethylaminoethoxybenzoyl)-benzofuran); CCCP, carbonyl cyanide 3-chlorophehylhydrazone; FDA, fluorescein diacetate; Hsf, heat shock factor; Hsp, heat shock protein; mt$\Delta\psi$, transmembrane potential on the inner mitochondrial membrane; PCD, programmed cell death; PI, propidium iodide; ROS, reactive oxygen species.

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Transcriptional activity of Msn2/Msn4p factors in *S. cerevisiae* cells is downregulated by both cAMP-dependent protein kinase A and the TOR (targets of rapamycin; a rapamycin-sensitive protein kinase) signaling system [4]. The system elevating expression of *HSP* genes by transcriptional activation of Hsfp is present in all studied species. It was thought for a long time that the Hsfp transcriptional activity in the absence of heat shock is suppressed due to downregulation caused by interaction between Hsp70p (heat shock protein 70) and Hsp90p (heat shock protein 90). This hypothesis supposed that the level of denatured proteins in the cell increases under the heat shock. The proteins Hsp70p and Hsp90p are released from their complex with Hsfp to react with denatured proteins, which results in activation of Hsfp [5]. However, recent data suggests that this hypothesis is too simple to describe all the diversity of factors influencing transcription activity of Hsfp [6]. It becomes obvious that reactive oxygen species (ROS) [7] and Ca^{2+} [8] can have a significant effect on Hsfp activation and, hence, on HSP expression in mammalian and plant cells.

In plant [8] and mammalian [9] cells heat shock causes a short-term increase in cytosolic Ca^{2+} level. One can expect that a similar effect can be also observed in *S. cerevisiae* [10]. Earlier, we demonstrated that exogenous

Ca^{2+} induces Hsp104p synthesis in yeast cells and elevates their thermotolerance [11]. This suggests that in yeasts, like in plants and mammals, Ca^{2+} plays an important role in both activation of *HSP* gene expression and development of acquired thermotolerance. To test this hypothesis, here we studied the effect of amiodarone (AMD), a substance elevating Ca^{2+} level at ordinary temperatures, on *HSP* synthesis and thermotolerance in the yeast *S. cerevisiae*. As previously reported [12-14], AMD causes a short-term elevation of Ca^{2+} level in the yeast cell cytosol.

MATERIALS AND METHODS

Strains and growth conditions. The yeast *S. cerevisiae*, parent type strain Ψ-74-D694 (*MATa*, *ade1-14(UGA)*, *trp1-289(UAG)*, *his3Δ-200*, *ura3-52*, *leu2-3*, *112* [*psi*$^-$]) and its isogenic mutant Ψ-74-D694::*hspΔ-1L* (*MATa*, *ade1-14(UGA)*, *trp1-289(UAG)*, *his3Δ-200*, *ura3-52*, *HSP104::LEU2)*, were kindly provided by Susan L. Lindquist (Whitehead Institute for Biomedical Research, USA); we also used the strain W303-1A (*MATa*, *ade2-1*, *ura3-1*, *his3-11*, *15 leu2-3*, *112 trp1-1*, *can1-100*, *SUC2*). The yeast cells were maintained at 30°C on the YEPD (yeast extract, peptone, dextrose) medium containing 0.5% yeast extract, 1% peptone, 2% glucose, and 1.5% agar-agar. The cells were grown at 30°C in 100-ml Erlenmeyer flasks with 25 ml of liquid YEPD or YEPE (yeast extract, peptone, ethanol; 2% ethanol was added instead of glucose), and cells in logarithmic growth phase were used in experiments.

Fluorescence microscopy. The count rates of live and dead cells were determined from the fluorescence of fluorescein diacetate (FDA) and propidium iodide (PI) following incubation of the cells for 2 min with these dyes taken at concentrations of 50 μM and 10 μg/ml, respectively. The inner mitochondrial membrane potential was qualitatively visualized using the potential-dependent cationic dye TMRM (tetramethylrhodamine methyl and ethyl esters) at the final concentration of 5 μM. The data were recorded following 10-min incubation of the cells with the dye. Fluorescence microscopy was carried out using an AxioObserverZ1 inverted microscope (Germany) equipped with an AxioCamMRm3 digital monochrome camera and software package AxioVisionRel.4.6.

Counting of colony-forming units (CFU). To count CFU, serial dilutions of yeast cells were seeded on agarized YEPD medium. Following 24-48-h incubation at 30°C, the number of colonies was counted.

Isolation of total protein and immunoblotting. The cells were centrifuged, washed thrice with distilled water, and stored at −70°C before experiments. The cells were thawed, resuspended in isolation buffer (0.1 M Tris-HCl, pH 7.4-7.6, containing 3 mM SDS and 1 mM β-mercaptoethanol), frozen in liquid nitrogen, and ground with quartz sand. Crude cell components were removed by centrifugation (15,000*g* for 15 min), and the protein was treated with three volumes of cold acetone. The pellet was washed thrice with acetone and dissolved in sample buffer (0.625 M Tris-HCl, pH 6.8, containing 8 mM SDS, 0.1 M β-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue). Protein concentration was determined by the Lowry method. Following SDS-PAGE in 12% polyacrylamide gel, immunoblotting was carried out using antibodies against Hsp104p (SPA-8040; Stressgen, USA) and against Hsp60p (US Biological H1830-77B, USA).

All experiments were repeated no less than three times. The data were statistically processed for the mean values and standard deviations.

RESULTS

Yeast cell death under mild heat shock following the treatment with AMD. AMD, which is used in clinical practice as an antiarrhythmic drug, is a potent fungicide. Taken at micromolar concentrations, it inhibits growth and causes death of *S. cerevisiae* cells [12-14]. To determine toxic effect of AMD on the yeast cells, we estimated their survival by counting colonies on a solid medium after 48-h growth at 30°C (Fig. 1a) as well as by staining with FDA and PI directly after incubation with AMD (Fig. 1b). FDA and PI stain live and dead cells, respectively [15].

In accordance with the literature, 60-min treatment with AMD resulted in death of *S. cerevisiae* W303-1A cells (Fig. 1), the level increasing with increasing AMD concentrations. Estimations of cell survival by CFU counting 48 h after the treatment (Fig. 1a) and by counting FDA-positive and PI-negative cells directly after the treatment (Fig. 1b) gave similar results. Some difference between these data was observed at AMD concentrations of 20 and 50 μM.

It was reported elsewhere [12] that AMD caused programmed cell death (PCD) of yeast cells due to increase in ROS production, DNA fragmentation, and cytochrome *c* release from mitochondria. Damaging heat shock can also lead to PCD of cultured plant cells. The hallmark of PCD in plants is time course of viability decrease [16, 17]. The cells remained alive directly after the heat shock and died after 24-48 h incubation at the normal temperature. So, to compare the character of cell death after treatment with AMD and under heat shock conditions, we studied yeast cell survival after heat shock of varied intensity.

The live cell count by the two methods described above demonstrated that incubation at both 39 and 42°C for 60 min has no effect on the yeast cell survival. Cell death began to be observed when the temperature was increased to 45°C (Fig. 2, a and b). However, in this case

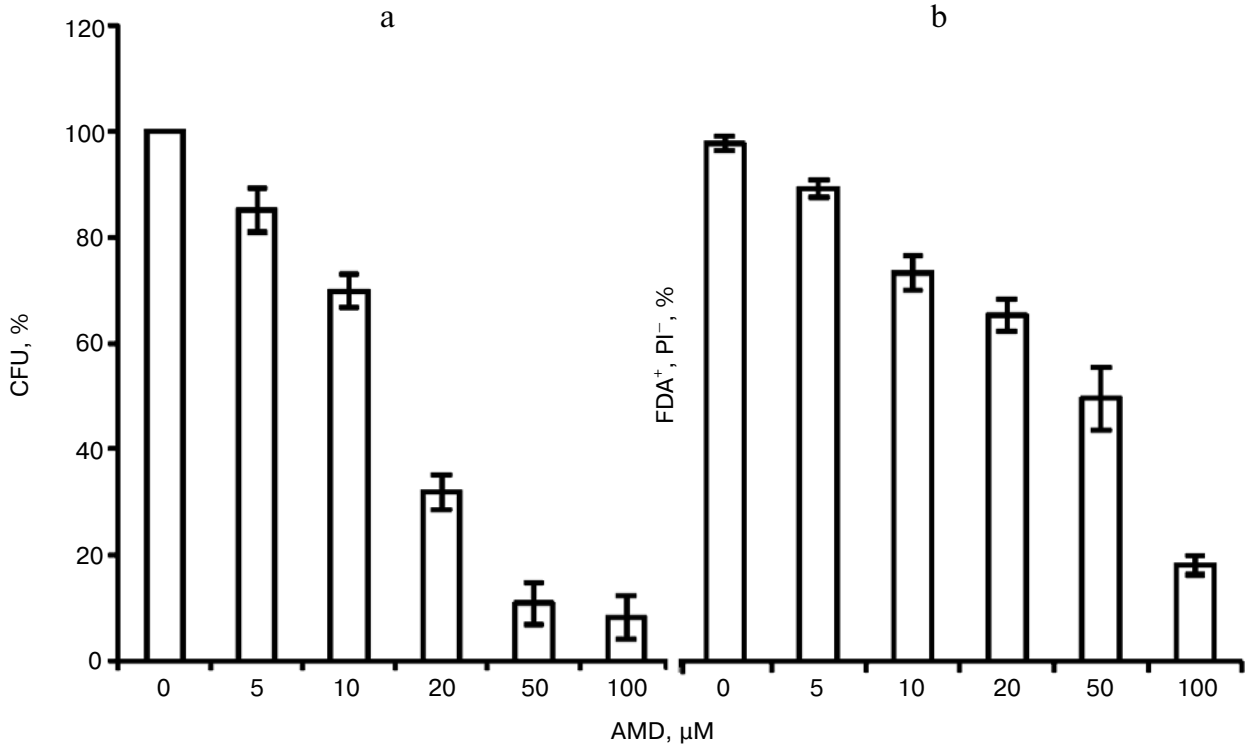

Fig. 1. Characteristics of *S. cerevisiae* cell death following treatment with AMD. Survival of the cells was estimated by CFU counting 48 h after the treatment with AMD (a) and by counting FDA-positive and PI-negative cells directly after the treatment (b).

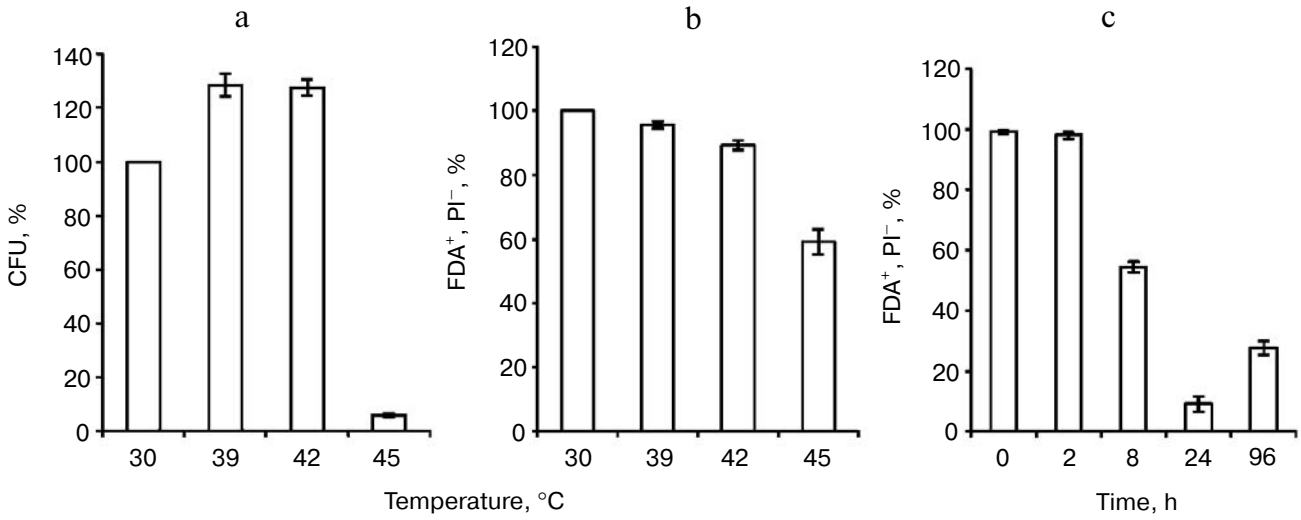

Fig. 2. Effect of different temperatures on survival of *S. cerevisiae* W303-1A cells. Cells cultured on YEPD medium were incubated for 60 min at 30, 39, 42, or 45°C. Survival was determined by CFU counting 24-48 h after the incubation (a) and by staining with FDA and PI (b). c) *Saccharomyces cerevisiae* Ψ-74-D694 cells were incubated at 45°C for 30 min, and survival was determined by staining with FDA and PI 0, 2, 8, 24, and 96 h after further incubation at 30°C.

some difference was seen in the data obtained by two methods. The CFU counting demonstrated that only 10% of the cells formed colonies (Fig. 2a), whereas >50% were stained with FDA as live directly after the heating (60 min at 45°C) (Fig. 2b). These data suggest the time progression of cell death induced by the heat shock at 45°C. To verify this, we subjected the *S. cerevisiae* Ψ-74-D694 cells to mild heat shock (45°C, 30 min) and moni-

tored percentage of live and dead cells during incubation (2-96 h) at 30°C by staining with FDA and PI. Directly after the heat shock 100% of the cells were stained as live (Fig. 2c). The same result was obtained 2 h after incubation at 30°C. Dead cells only appeared after 8 h incubation, and their share reached 90% after 24 h. Following 96-h incubation the level of live cells began to increase, suggesting that the cells outliving the stress had begun to divide. Thus, the data has shown that, like cultured plant cells [16, 17], yeast cells die with time following mild heat chock at 45°C.

AMD induces synthesis of Hsp104p. Mild heat shock causes a short-term elevation of Ca^{2+} level in cells of plants [8] and animals [9]. It is likely that a similar event also occurs under heat shock in *S. cerevisiae* [10]. It is arguable that elevation of intracellular Ca^{2+} is in causal connection with expression of the *HSP* genes [8, 9]. It is known that AMD leads to increase in Ca^{2+} concentration in the yeast cell cytosol [12-14]. The treatment with AMD at 30°C for 60 min induced Hsp104p synthesis that was more prominent with increase in concentration of AMD (Fig. 3a). A distinct increase in amount of Hsp104p was observed at AMD concentrations of 20, 50, and 100 μM. The induction of Hsp104p synthesis following heat shock at 39°C was very much more than that following the treatment with AMD taken at any of the tested concentrations (Fig. 3a).

Despite the fact that AMD produced lethal effect on the yeast cells (Fig. 1), it induced Hsp104p synthesis under the same conditions (Fig. 3a). Hence, the yeast cells dying from AMD continue to express Hsps. As we showed earlier on cultured *Arabidopsis thaliana* cells, induction of various Hsps, such as Hsp101p, Hsp70p, and Hsp17.6p (class I) and Hsp17.6p (class II) was only observed on heating causing no negative effect on survival [17]. This suggests that activation of *HSP* expression and death under severe heat shock are mutually exclusive. To make sure this suggestion is correct for the yeast cell, we performed a similar experiment. One can see in Fig. 3b that Hsp104p synthesis is induced at 39 and 42°C, while elevation of the temperature to 45°C inhibits synthesis of this protein. The data presented in Fig. 2a indicates that the temperatures 39 and 42°C had no effect on the yeast cell survival, whereas incubation at 45°C significantly decreased it. Thus, induction of Hsp104p synthesis in yeast is suppressed when the temperature high enough to affect their survival.

AMD elevates yeast thermotolerance that depends on the presence of Hsp104p. Protein Hsp104p plays a crucial role in development of yeast cell thermotolerance [3]. Since AMD induces Hsp104p synthesis, we studied how this substance influences the yeast cell ability for opposing the effect of severe heat shock. To do this, we used the parent strain Ψ-74-D694 and its isogenic *HSP104* mutant (*hsp104Δ*) characterized by absence of Hsp104p synthesis at 39°C (Fig. 4a). In these experiments AMD was used at concentration 20 μM. The yeast cells were treated with AMD at 30°C for 30 min, washed, and subjected to severe heat shock at 50°C. Previously, we had found that AMD itself did not affect the cell viability of the tested strains (data not shown). As follows from Fig. 4b, pretreatment with AMD led to drastic increase in thermotolerance of the parental strain. Under the same conditions, thermotolerance of *hsp104Δ* mutant also increased, but to a far less extent. Thus, both AMD and mild heat stress protect the yeast cells from death under severe heat shock. Moreover, this effect depends on the presence of Hsp104p.

CCCP induces synthesis of Hsp104p and elevates thermotolerance of yeast cells. We previously reported that under heat stress mitochondrial inhibitors and uncouplers suppressed both induction of Hsp synthesis and development of acquired thermotolerance in plant [17] and yeast [18] cells. However, at the ordinary temperature these inhibitors in some cases could elevate thermotolerance and induce synthesis of Hsps [17, 18]. Like AMD, the classical uncoupler CCCP (carbonyl cyanide 3-chlorophehylhydrazone) is known to elevate Ca^{2+} concentration in *S. cerevisiae* cells [19].

Taken at concentration of 20 μM, CCCP inhibited induction of Hsp104p synthesis caused by mild heat stress (39°C) (Fig. 5a), thus supporting previously reported data

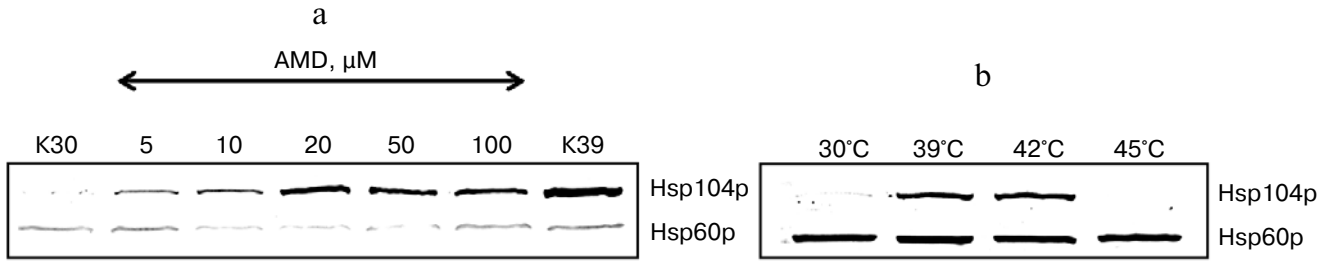

Fig. 3. Effects of AMD and different temperature on Hsp104p synthesis. a) Induction of Hsp104p synthesis following treatment with AMD. *Saccharomyces cerevisiae* W303-1A cells were treated with 0-100 μM AMD at 30°C for 60 min (K30 – control). For comparison, synthesis of Hsp104p and Hsp60p at 39°C for 60 min (K39) is shown on the right. b) Synthesis of Hsp104p and Hsp60p following the incubation of yeast cells for 60 min at different temperatures.

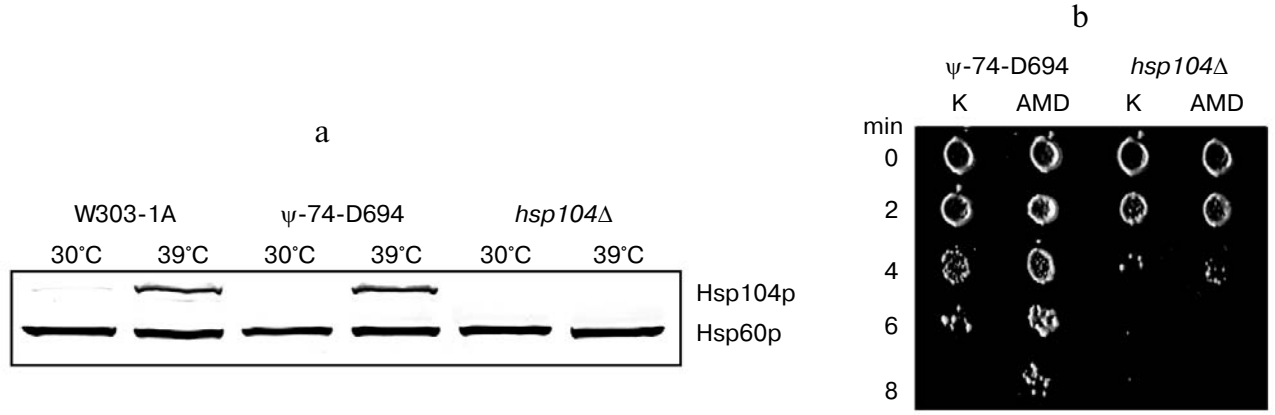

Fig. 4. Effect of AMD on synthesis of Hsp104p and thermotolerance of *S. cerevisiae* cells. a) Synthesis of Hsp104p and Hsp60p under mild heat stress (39°C). b) Survival of the cells after a severe heat shock (50°C). The Ψ-74-D694 (parental type) and *hsp104Δ* mutant cells were incubated with 20 μM AMD for 30 min at 30°C, washed free from AMD, and subjected to the heat shock (50°C).

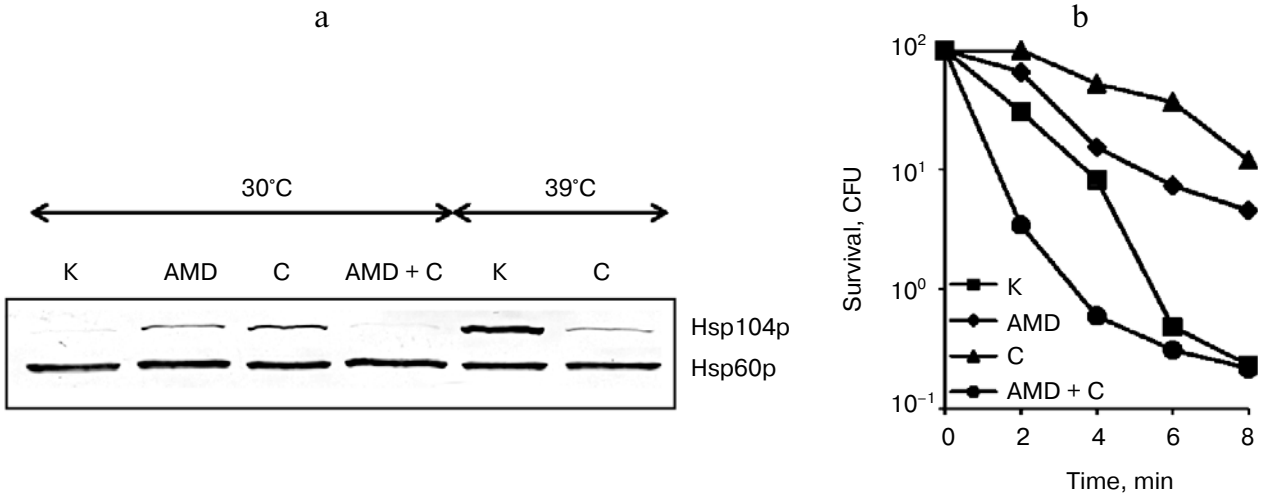

Fig. 5. Effect of AMD and CCCP on thermotolerance and synthesis of Hsp104p in *S. cerevisiae*. Cells were treated for 30 min at 30 or 39°C either in the absence of additives (K) or in the presence of 20 μM AMD, 20 μM CCCP (C), or 20 μM AMD plus 20 μM CCCP (AMD + C). a) Synthesis of Hsp104p and Hsp60p. b) Survival after heat shock (50°C) determined from CFU number.

[18]. At 30°C both 20 μM CCCP and 20 μM AMD taken separately considerably elevated Hsp104p synthesis, but their combined effect was negligible. A similar result was obtained when we studied thermotolerance of yeasts treated with CCCP and AMD at 30°C and then subjected to severe heat shock at 50°C. CCCP and AMD taken separately considerably increased survival of the yeast cells, whereas their combination decreased thermotolerance below that of the control (Fig. 5b).

AMD elevates fluorescence of TMRM. It was shown using cultured cells of mammals [9], plants [17], and yeasts [18] that mild heat stress elevates the transmembrane potential on the inner mitochondrial membrane (mtΔψ). AMD caused a similar increase in mtΔψ in *S. cerevisiae* cells as determined from change in fluorescence of potential-dependent probes TMRM [12] and JC-1 [20]. In accordance with the literature data [12, 20], the treatment of *S. cerevisiae* W303-1A cells with AMD for 10 min at 30°C led to increase in TMRM fluorescence, and this was more prominent as the AMD concentration was increased (Fig. 6a). These data suggest that AMD elevates mtΔψ in *S. cerevisiae* cells.

Saccharomyces cerevisiae is a facultative aerobe that meets ~75% of its energy requirements by fermentation (when grown on glucose). Under these conditions its respiration is lowered, although not completely repressed. When grown on non-fermentable carbon source, particularly ethanol, yeast cells obtain energy only by oxidative phosphorylation [21]. Because of this TMRM fluorescence of yeast cells was significantly lower when grown on

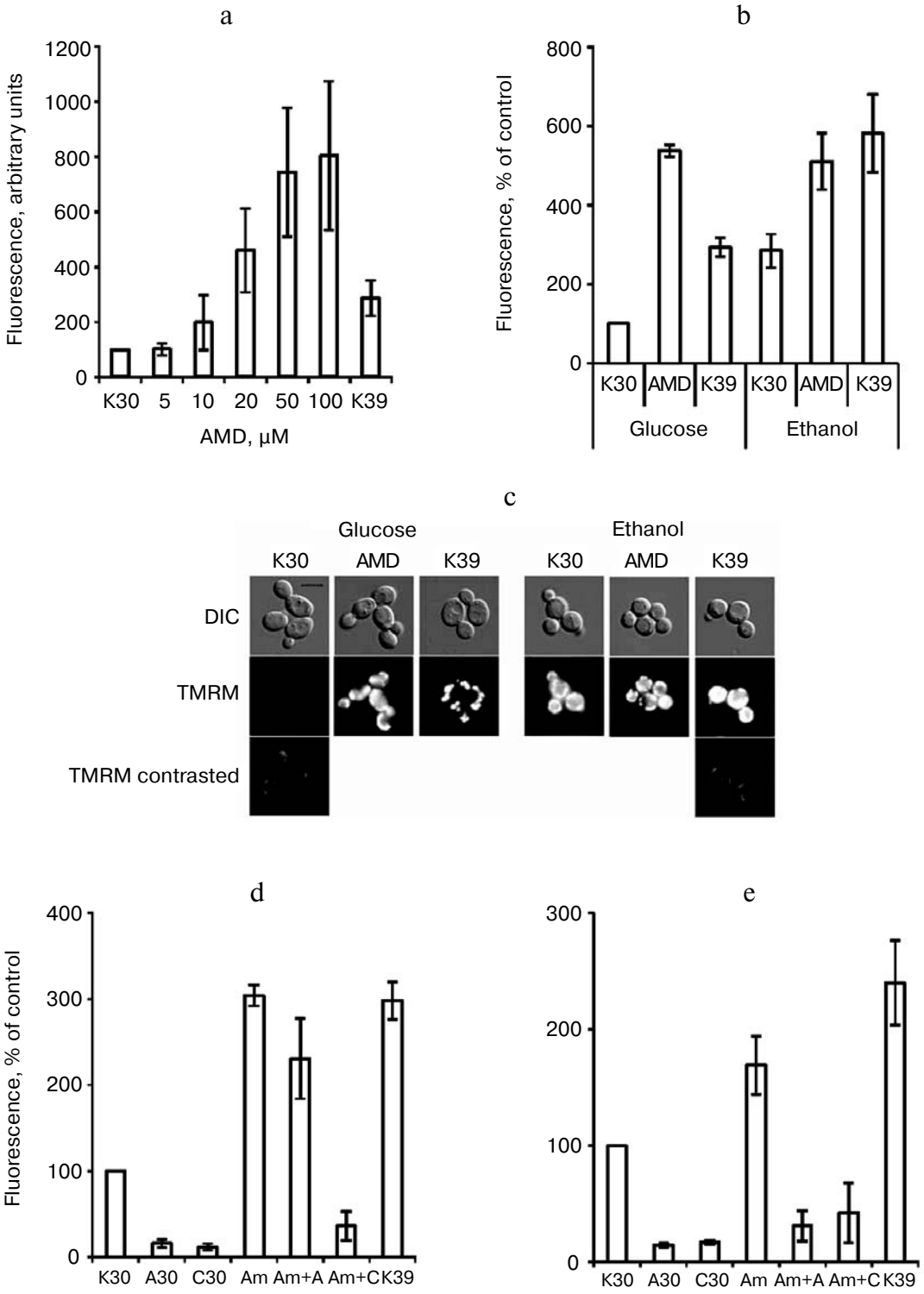

Fig. 6. Effect of AMD on TMRM fluorescence in yeast cells. a) Dependence of TMRM fluorescence level in *S. cerevisiae* W303-1A cells on concentration of AMD. The cells were treated with 0-100 μM AMD for 60 min at 30°C on YEPD medium. b) Intensity of TMRM fluorescence (c) following incubation of the cells in presence of 20 μM AMD or without it at 30°C (K30) or 39°C (K39) in YEPD (containing glucose) or YEPE (containing ethanol) medium. DIC, differential interference contrast microscopy. d, e) Effects of AMD, antimycin A, and CCCP on the TMRM fluorescence of yeast cells depending on the energy source. *Saccharomyces cerevisiae* W303-1A cells were incubated for 10 min at 30 or 39°C in the absence (K30 and K39) or in the presence of 20 μM AMD (Am), 10 μM antimycin A (A30), 20 μM CCCP (C30), 20 μM AMD and 10 μM antimycin A (Am + A), or 20 μM AMD plus 20 μM CCCP (Am + C).

the medium with glucose than on the medium with ethanol (Fig. 6, b and c). Similar data were reported earlier by other authors who showed that fluorescence of another potential-dependent dye, rhodamine 123, depends on whether oxidative or fermentative metabolism is occurring in yeasts [22]. During fermentation, mtΔψ in yeasts can be maintained due to the electrogenic transport of $(ATP)^{4-}$ in exchange for $(ADP)^{3-}$ across the inner mitochondrial membrane by adenine nucleotide translocator [23]. Elevation of TMRM fluorescence in yeast cells grown on ethanol as the energy source suggests that alteration of TMRM fluorescence correctly reflects alteration of mtΔψ.

AMD and heat stress elevated TMRM fluorescence independently of the energy metabolism type. AMD at concentration 20 μM caused about the same elevation as mild heat stress at 39°C (Fig. 6, b and c). The protonophore CCCP effectively suppressed elevation of TMRM fluorescence induced by AMD independently of the energy metabolism type (Fig. 6, d and e). However, antimycin A insignificantly inhibited elevation of TMRM fluorescence on glucose-containing medium (Fig. 6d), but its inhibitory effect was the same as that of CCCP on ethanol-containing medium (Fig. 6e). Thus, we suppose that mechanisms of mtΔψ elevation in response to AMD differ depending on the energy metabolism type. Earlier, we obtained a similar result when we studied the effect of heat shock on mtΔψ [18].

DISCUSSION

In accordance with the literature [12-14], AMD decreases yeast viability (Fig. 1). The mechanism of AMD toxicity is not completely understood. AMD is known to induce a short-term elevation of Ca^{2+} concentration in yeast cell cytosol [12-14]. Since excessive elevation of internal Ca^{2+} level can result in cell death, the toxicity of AMD is supposed to be associated with the elevation in concentration of cytosolic Ca^{2+} up to its dangerous level [13]. In fact, a direct correlation has been shown between the ability of AMD to elevate cytosolic Ca^{2+} level and its lethal effect on the yeast cell [13, 14]. However, this point of view is in disagreement with the results suggesting substantial elevation of AMD toxic effect on yeast due to inhibition of Ca^{2+} influx into the cytosol by Ca^{2+} chelators, and on the contrary, a protective effect of exogenous Ca^{2+} [13]. Hence, when cells are treated with AMD the elevation of Ca^{2+} content in their cytosol is necessary for elevation of yeast resistance against the stress. It is very likely that both points of view are correct. Calcium ion plays a dual role in the cells of any organism [24]. The elevation of Ca^{2+} concentration above its certain critical level leads to cell death. On the other hand, Ca^{2+} induces an adaptive reaction and development of stress resistance.

It is obvious that, under the conditions of our experiments, the mechanism of yeast cell death following the treatment with AMD differs from that under mild heat shock. The used AMD concentrations caused rapid cell death. This is supported by the data on cell viability after 24-h incubation (Fig. 1a) and immediately after the treatment with AMD (Fig. 1b). However, yeast cell death after mild heat shock develops with time (Fig. 2c).

Despite its toxic effect, AMD induces synthesis of Hsp104p in *S. cerevisiae* cells (Fig. 3a). On the other hand, heat shock inducing the yeast cell death (45°C) did not result in induction of Hsp104p synthesis (Fig. 3b). *Arabidopsis thaliana* cells also did not synthesize Hsps when elevation in heat treatment intensity decreased their viability [17]. Thus, in the case of treatment with AMD a reverse feedback is absent between Hsp synthesis in yeast cells and their death. Possible causes of this difference might be the different dynamics of cell death under treatment with AMD (Fig. 1) and heat shock (Fig. 2). Nevertheless, the treatment with AMD at a concentration that does not lead to cell death not only induced the synthesis of Hsp104p, but also increased thermotolerance of the cells. Development of thermotolerance under AMD treatment also depended on the presence of functional Hsp104p (Fig. 4b), additionally supporting the leading role of this protein in development of yeast thermotolerance.

Treatment with exogenous Ca^{2+} induces Hsp synthesis and elevates thermotolerance in yeasts [11] and plants [8]. A similar effect can be achieved by treatment of the yeast with AMD or the typical uncoupler CCCP (Fig. 5). AMD is well known to induce a short-term elevation of cytosolic Ca^{2+} content in *S. cerevisiae* [12-14]. CCCP has a similar effect [19]. Thus, the results support the supposition [11] that calcium can play an important role in activation of *HSP104* expression and development of yeast ability to resist the damaging effect of heat shock.

As we mentioned above, *HSP104* gene expression under heat stress is regulated by Hsfp and Msn2/Msn4p transcriptional factors [3]. It was shown that exogenous Ca^{2+} and AMD activate expression of 54 genes in *S. cerevisiae* cells, most of them being under the control of Msn2/Msn4p [25]. We suppose that the ability of AMD to induce Hsp104p synthesis (Figs. 3a and 5a) depends on Msn2/Msn4p functioning, and the activity of these transcriptional factors depends on the presence of Ca^{2+}. The latter supposition is confirmed by data demonstrating the activation of Msn2/Msn4-dependent expression of the *S. cerevisiae GPX1* gene encoding glutathione peroxidase under treatment with $CaCl_2$ [26].

AMD causes transient elevation in the level of yeast cytosolic Ca^{2+} [12-14]. AMD also induces TMRM fluorescence increase in yeast cells, which may suggest the elevation of mtΔψ (Fig. 6). The ability of AMD to hyperpolarize the yeast inner mitochondrial membrane has been demonstrated earlier [12, 20]. The authors of those works supposed that elevated Ca^{2+} content stimulates

activity of respiratory enzymes and elevates the coupling of respiration and energy production, thus resulting in elevation of mtΔψ.

As a rule, the increase in cytosolic Ca^{2+} in mammalian cells is accompanied by the elevation of mtΔψ [24]. This is because mitochondria serve as a store for intracellular Ca^{2+} in mammalian cells. Transport of Ca^{2+} into the mitochondria is determined by the value of mtΔψ. Being inside the mitochondria, Ca^{2+} activates pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and NAD^+-isocitrate dehydrogenase. This, in turn, stimulates respiration, increases ATP production, and, as a result, elevates mtΔψ. In contrast, none of the listed enzymes are activated by Ca^{2+} in *S. cerevisiae* [27]. Moreover, the system of active transport of Ca^{2+} was not found at all in *S. cerevisiae* mitochondria [28]. Hence, it remains unknown whether elevation of Ca^{2+} level in the *S. cerevisiae* cell can influence activity of mitochondria. So, the data on the effect of AMD on elevation of mtΔψ in *S. cerevisiae* cells require further careful consideration and confirmation by other studies.

Nonetheless, some literature data suggest that an association is still exists between alteration of intracellular Ca^{2+} level and mitochondrial function in *S. cerevisiae* cells. As we noted above, the classical mitochondrial uncoupler CCCP caused elevation of Ca^{2+} concentration in yeast cytosol. Glycerol had the same effect, and antimycin A inhibited the effect of glycerol on the Ca^{2+} level [19]. Deletion of the *PMR1* gene encoding Ca^{2+}/Mn^{2+} ATPase increased intracellular Ca^{2+} concentration [14] and simultaneously affected respiration [29]. It is likely that in *S. cerevisiae* cells Ca^{2+} can indirectly influence the mitochondrial activity by modulating the TOR signaling system. This system is known to influence activity of mitochondria in yeasts and depend on the presence of Ca^{2+} [30]. This supposition is confirmed by the fact that the patterns of *S. cerevisiae* gene expression in response to AMD, exogenous Ca^{2+}, and rapamycin (an inhibitor of the TOR signaling system) are quite similar [25]. When analyzing mechanisms by which the intracellular Ca^{2+} can influence the activity of yeast mitochondria, one must take into account possible contacts between the mitochondrion and endoplasmic reticulum, which can facilitate transduction of Ca^{2+}-signal [31]. Thus, we cannot exclude that elevation of Ca^{2+} in response to AMD might influence indirectly rather than directly the activity of yeast mitochondria and, correspondingly, the change in mtΔψ.

Like AMD, heat stress at 39°C leads to increase in TMRM fluorescence in *S. cerevisiae* cells (Fig. 3, see [18]), as well as in cultured cells of *A. thaliana* [17]. Similar data were obtained using mammalian cell culture [9]. The authors of work [9] showed that both heat shock and agents causing lipid alterations in plasma membrane lead to elevation of Ca^{2+} level in cytosol and hyperpolarization of the inner mitochondrial membrane, which is accompanied by expression of *HSP* genes. It was hypothesized [9, 17, 18] that elevation of mtΔψ can be the early response of the cell to stress, which leads to further activation of *HSP* gene expression.

The data of the present work confirm this hypothesis. There is a direct correlation between elevation of TMRM fluorescence and induction of Hsp104p synthesis in *S. cerevisiae* cells under treatment with AMD and under heat shock. Elevation of mtΔψ in mitochondria of mammalian [32] and yeast [12] cells correlates with elevation of ROS production. At the same time, it is likely that elevation of ROS production is an important element in activation of *HSP* gene expression in yeasts [33] and plants [7]. The elevation of Ca^{2+} level might indirectly stimulate activity of yeast mitochondria and elevate mtΔψ. Elevation of mtΔψ leads to enhancement of ROS production, which, in turn, activates *HSP* gene expression. It is worth noting that a tight association exists between the change in intracellular Ca^{2+} level and ROS production in the living cell [34].

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