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CHANGES IN U937 CELL VIABILITY INDUCED BY STRESS FACTORS - POSSIBLE ROLE OF CALMODULIN

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Current studies were aimed to elucidate influence of magnetic field (MF) stimulation on cell viability and its effect on expression of calmodulin (CaM) and Hsp70 protein which plays a role of cell stress indicator and is a Ca^{2+}-dependent CaM-binding protein. For the experimental model we have chosen U937 cell line exposed to chemical- and/or physical stress factors. Puromycin (PMC) was used as a chemical apoptosis inducer. Alternating (AC) (6.5_{rms} mT, 35 Hz) magnetic field combined with 6 mT static (DC) component, or pulsed electromagnetic field (45 ± 5) mT, 50 Hz (PEMF) acted as physical stressors. Cell viability was assessed by flow cytometry, and the Western blot analysis was carried out for CaM and Hsp70 levels in cytosolic extracts of U937 cells. Cell viability in samples exposed to MF alone did not differ from sham sample, for both types of MF exposure systems. Simultaneous action of MF and PMC influenced cell viability in type of MF stimulation-dependent manner. In contrast to PEMF + PMC stimulated samples, combination of ACDCMF with PMC enhanced cell death compared to PMC control. The observed changes in cell viability were correlated with changes in level of CaM and Hsp70 proteins. Immunoblots have shown, that cytosolic content of both CaM and Hsp70 proteins was enhanced in PMC-treated sample, and further elevated for ACDCMF + PMC. For PEMF + PMC stimulated samples, level of CaM was reduced compared to PMC-treated sample. The results suggest that the changes in expression of CaM and CaM-dependent proteins might modulate effectiveness of cell death under stimulation with MF and/or cytotoxic agents.

K e y   w o r d s : *calcium binding proteins, intracellular calcium ions level, calmodulin leukemic cells, magnetic fields, cytotoxic agents, protein kinase C*

INTRODUCTION

Numerous studies have shown that various stress factors (e.g. chemically derived cytotoxic agents, physical factors) can influence normal cell function. According to some experimental results, one of the cell stressors may be exposure to magnetic fields (MFs). Because of the wide distribution of MF sources in the environment, magnetic fields have recently been intensively examined especially after not consistent reports considering their possible carcinogenic- or therapeutic role (1-4). The observed bio-effects remain controversial as the mechanism(s) underlying the effects of MF action on cells are still not known, in particular, non-thermal effects have not been fully recognized (5-8). The most often considered options are radical pair mechanism and related changes of reactive oxygen species (ROS) chemistry which can occur even for very weak MF (9-12), and modulation of intracellular ions mobilization (13-15).

Nevertheless, the biophysical models proposed so far, did not quantitatively describe and incontestable link all observed bio-effects with specific intracellular target(s) of MF. Interestingly, caused bio-effects vary depending on cell types and the parameters of MF (e.g. magnetic flux density, frequency, signal waveform, time of exposure) itself (5, 16). It is also experimentally verified, that significant bio-effects (e.g. changes in cell viability) have been observed for simultaneous action of MF and cytotoxic drugs (17-19). This scenario could be fulfilled when energy of used MF is not enough to cause direct lethal lesions, thus cytotoxic agent must be used to decrease the 'natural cellular defense mechanisms'. To this day, mechanism(s) of action of various cytotoxic drugs were tested on different cell types in a time- and dose-dependent manner (20-22). Synergic effects induced by chemical- and physical factors, may lead to cell dysfunction and/or cell death.

A multitude of cellular processes are controlled and regulated by Ca^{2+} ions (23, 24), thus it is not surprising that a leading hypothesis concerning action of exogenous stressors on living cells is related to the disturbance of Ca^{2+} homeostasis and Ca^{2+}- triggered signalling pathways (16, 25-27). Indeed, numerous studies performed on various cell model systems have revealed relation between cell response to stress factor(s) and changes in the intracellular Ca^{2+} calcium level. For instance, in (28) authors have found changes in cytosolic and endoplasmic calcium content in U937 cells due to PMC or topoisomerase II inhibitor etoposide (VP16) action, leading to apoptotic cell

death. However, the cell response can differ depending on whether the calcium signal develops as a local or global change, cell types, internal condition of the cell system and combination of stressors. The diversity of cell responses (e.g. viability versus intracellular calcium level) for the same stressors (e.g. PMC, etoposide and/or DCMF) was well-documented in (29), where authors have compared several primary cultures and cell lines. The Ca^{2+}- mediated changes in various cell types including role of calmodulin were elaborated in (30).

Since the modulation of Ca^{2+}- dependent signalling pathways at different stages is suggested as one of many hypothetical mechanisms underlying cell response to various stress factors, it was verified here for simple experimental model.

Current studies were aimed to elucidate influence of low-frequency magnetic field stimulation on cell viability and its effect on expression of calmodulin (CaM) - the most important calcium binding protein, and Hsp70 protein which plays a role of cell stress indicator and belongs to Ca^{2+}- dependent-CaM-binding proteins.

The presented studies were focused not only on bio-effects triggered by exposure to MF alone, but also in combination with chemical stressor (synergic effect). As an inducer of apoptosis, puromycin (PMC) has been chosen, as it plays a multiple role in cell *via* inhibition of translation in protein synthesis process or *via* acting as ER Ca^{2+} leak channel opener and an ER stressor (22, 26).

MATERIALS AND METHODS

Cell culture

U937 cells (human leukemia cell line; ATCC, Rockville, MD) were cultured in RPMI 1640 medium (Gibco-BRL, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-BRL, USA), 0.2 M L-glutamine (diluted 100 times) and 50 mg/ml gentamicin (diluted 1000 times) (Sigma-Aldrich, Germany) at 37°C in 5% CO_2 incubator of 90% humidity. These conditions were found optimal for U937 cells growth, and cells were passaged every four days. Cell viability was monitored by trypan blue exclusion method and counted with a haemocytometer. The experiments were performed on viable cells ($\geq$ 98% viability) in the logarithmic phase of growth. For experiments with ACDCMF or PEMF, cells were seeded into 6-well- and 96-well culture plates to reach a final density of 2.5×10^5 cells/ml, respectively. In order to place cells in ACDCMF exposure device (2.5 h/day in threefold repetition), cells were transferred from plates to BD Falcon polystyrene test tubes. For PEMF stimulation culture plates were set directly in the apparatus pocket. Cells were harvested by centrifugation and washed with fresh medium after each stimulation. Procedure was verified to obtain the best accuracy and reproducibility. Cell death was induced with 50 µg/ml or 100 µg/ml (final concentration) of puromycin (PMC) added simultaneously with MF (3×2.5 h), in 24 h interval between repetitions. Control group of cells, cells after PMC and/or MF treatment were collected for flow cytometry and Western blot analysis.

Magnetic field exposure system

Cells were exposed to homogenous 6 mT static magnetic field (DCMF) combined with perpendicularly oriented sinusoidal component ACMF (35 Hz, 6.5_{rms} mT) or to (45 ± 5) mT, 50 Hz pulsed electromagnetic field (PEMF). The MFs were produced by two separate exposure systems. The details of the applied experimental apparatus are given elsewhere (17, 18). Briefly, the AC coil was fed from a signal generator (Agilent 33120A, Hewlett-Packard, USA) and an amplifier (Magnoter D56, MARP

Electronic, Poland). The DC coils arranged in series were fed from stabilized DC power supply (Unitra, Poland). The signals were first calibrated, allowing to collect calibration curves for static- and alternating components of the magnetic field. The homogeneity of the MF in the sample volume was tested and ensured with the accuracy of a few% by using a Gaussmeter (F.W. Bell 6010, Bell Technologies, USA). The MF- exposed samples and control samples were maintained in an incubator at conditions 5% CO_2 and 37.0 ± 0.1°C. Control samples were set in area free of MFs produced by exposure devices. The background MF for static- and alternating component was as follows: $< (0.05 \pm 0.01)$ mT and $< (0.03 \pm 0.01)_{rms}$ mT, respectively. Cell exposure to ACDCMF or PEMF was repeated three times (2.5 h per each day), in 24 h intervals between repetitions.

The choice of parameters for ACDCMF and PEMF was related to the following reasons: their proximity with parameters used in magnetotherapy and with the most abundant power line frequency; low-frequency signalling transduction in human body; and occurrence of non-thermal effects.

Western blot

Cytosolic extracts of cells were isolated using the following procedure: U937 cells 24 hours after last stimulation with PMC and/or MF were collected in ice-cold PBS by centrifugation (1200 rpm for 12 min. at 4°C) and washed in PBS twice. Cell pellet was lysed in 100 µl or 1 ml of cell lysis buffer (Invitrogen, USA) supplemented with 1 mM PMSF (Sigma-Aldrich, Germany) and protease inhibitor cocktail (Sigma-Aldrich, Germany) for 30 minutes, on ice. The samples were vortexed by 10 seconds at 10 minutes intervals. Next step, extracts were transferred to microcentrifuge tubes and centrifuged at 13,000 rpm for 10 minutes at 4°C. Supernatants were collected to further validation for proteins content using spectrophotometric measurement of absorbance at 280 nm (NanoDrop1000) and device for electrophoresis (Invitrogen, USA). Equal amount of lysate for each sample and molecular weight Novex® Sharp Protein Standard (Invitrogen, USA), were separated in 4 – 12% Bis-Tris GelNuPAGE® electrophoresis (40 min), using MES SDS running buffer (Invitrogen, USA). Separated proteins were dry blotted onto nitrocellulose iBlot®gel transfer stacks in iBlot Gel transfer device for 7 minutes. The nitrocellulose membrane was blocked with WesternDot™ blocking buffer (Invitrogen, USA) overnight at 4°C. Blocking procedure was followed with overnight at 4°C exposure to mouse antihuman monoclonal primary antibodies against calmodulin (CaM) (Invitrogen, USA) and against Hsp70 (RD Systems, USA), and beta-actin loading control antibody (Invitrogen, USA) as a control of immunoblotting. The antibodies were diluted 1:500, 1:4000 and 1:1000 at final concentration, respectively. According to manufacturer's procedure detection step was developed with Western blot detection kit (Invitrogen, USA), goat antimouse secondary polyclonal antibody (diluted 1:4000 at final concentration) with alkaline phosphatase and substrat Fast BCIP/NBT (Sigma-Aldrich, Germany). The obtained signal from protein detection was examined under VIS light using Transluminator equipped with PhotoCapt software (Vilber Lourmat, France). Quantitative protein levels were assessed by analysis of the grayscale level with the use of freely available software for image analysis (ImageJ 1.45s, USA). The relative band densities were normalized to appropriate β-actin bands used as reference protein.

Flow cytometry

Percentage of viable/non-viable cells was estimated with a BD FACSCalibur (San Jose, CA) flow cytometer equipped with CellQuest Pro software (BD, San Jose, CA), 24 h after last

stimulation with cytotoxic agent and/or MF. Cells were stained with Annexin V-APC labeled (AnV-APC, BD Biosciences, USA) and with 7-amino-actinomycin D (7-AAD; BD Biosciences, USA) according to the manufacturer's protocol. The details of the applied experimental procedure are given elsewhere (31, 32). AnV-APC – 7-AAD assay allowed to distinguish between viable cells and cells undergoing cell death (damaged cells). Annexin V-APC positive and double-positive (Annexin V-APC and 7-AAD) cells were analyzed as damaged ones. Viable population was registered as Annexin V-APC and 7-AAD negative. Unstained cells and cells stained with AnV-APC or 7-AAD alone were used as controls to adjust the fluorescence compensation settings at first. Number of events collected for each sample was $\geq 10^4$ to obtain reliable statistics.

Statistical analysis

Statistical evaluation of the experimental data was performed with U-Mann Whitney and Kruskal-Wallis tests considering P < 0.05 as the minimum level of significance (Statgraphics Centurion XVII). Data are presented as ratios, mean values, and standard deviation (S.D.) of three independent experiments, each performed at least in duplicate.

RESULTS

Changes in U937 cells viability and modulation of CaM and Hsp70 levels upon exposure to ACDCMF or PEMF alone, or when combined with 50 µg/ml PMC are shown in *Figs. 1 - 3*. Viability coefficient S, was assessed as a ratio of viable cells in the sample exposed to MF and/or PMC to viable cells in the relevant control sample (sham one (C) or only puromycin treated (PMC)). As shown in *Fig. 1* magnetic fields alone did not exert significant changes in cell viability, regardless of type of the applied MF, which stays in accordance with our previous reports (17, 18). Viability coefficient S was close to 1, for both types of MF exposure. Application of PMC has reduced percentage of viable cells in the treated sample about twofold in comparison to sham one.

However, simultaneous action of MF and PMC significantly influenced the U937 cell viability in type of MF- dependent manner. Applied ACDCMF enhanced lethal changes caused by cytotoxic agent (S was equal to 0.67 ± 0.05), while for PEMF the effect was opposite (S = 1.1 ± 0.01) indicating on its likely protective role against PMC- induced cell death. The applied here lower dose of cytotoxic agent influenced the effectiveness of cell death process in non-linear manner compared to our previous results, while maintaining the same trend of changes in cell viability. These results indicate on variety of possible cell responses to applied parameters of MF itself and/or intensity of chemical stressor.

The data in *Figs. 2* and *3* have shown, the differences in the amount of CaM and Hsp70 proteins between control-, PMC and/or MF stimulated samples. Observed changes in levels of expression of these proteins correspond with the effectiveness of cell death process, indicated by viability coefficient S (*Fig. 1A*, *1B*). Examination of immunoblots developed with anti-CaM antibody have shown, that detected amount of CaM has been increased in PMC-treated samples about 35% compared to control ones. In case of samples simultaneously stimulated with PMC and MF, the level of CaM was further elevated (0.36 ± 0.02) (*Fig. 2A*) or reduced (0.19 ± 0.01) (*Fig. 2B*) in dependence on the type of applied MF. There was no significant differences in the expression level of calmodulin between control- and only MF-treated samples for both types of magnetic exposure.

The same tendency of lesions in the detected amount of CaM was preserved for higher dose (100 µg/ml) of PMC and/or ACDCMF, or ten times higher volume (1 ml) of cell lysis buffer (not shown on the graph). In this case the detected amount of CaM was in the range of (0.32 ± 0.01 ÷ 0.38 ± 0.01) for PMC-treated samples, and has been elevated to (0.47 ± 0.01 ÷ 0.51 ± 0.01) for samples simultaneously stimulated with ACDCMF and PMC. The lower values (in the brackets) of CaM content are related to the samples lysed in 1 ml of cell lysis buffer instead of 100 µl. The two different volumes of the reagent were used to verify the reproducibility of the observations in accordance with the applied methodology. Because the tendency of changes was preserved in both cases, the observations are rather attributed to the intracellular mechanisms not to the technical details.
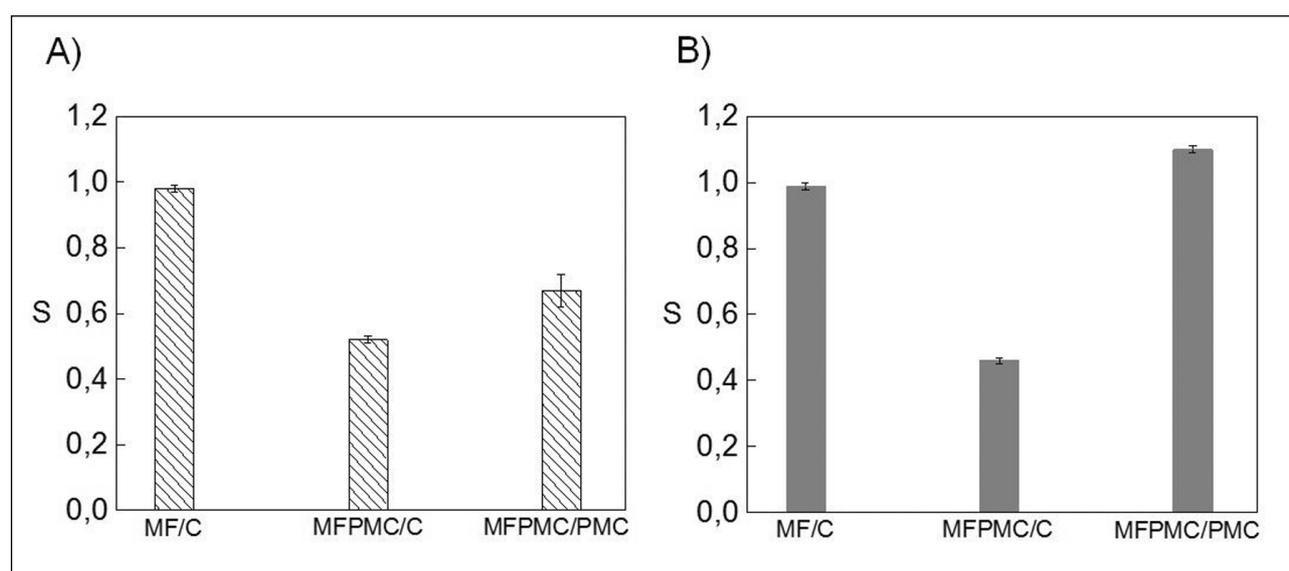
Fig. 1. Changes of viability coefficient S in accordance with applied MF. (A) ACDCMF (6 mT + 6.5 $_{rms}$ mT; 35 Hz); (B) PEMF (45 ± 5 mT; 50 Hz). C, control sample; PMC, U937 cells treated with puromycin; MF, MF exposed cells; MFPMC, MF and PMC stimulated cells. Data are expressed as mean ± S.D. All differences are statistically significant (P < 0.05).
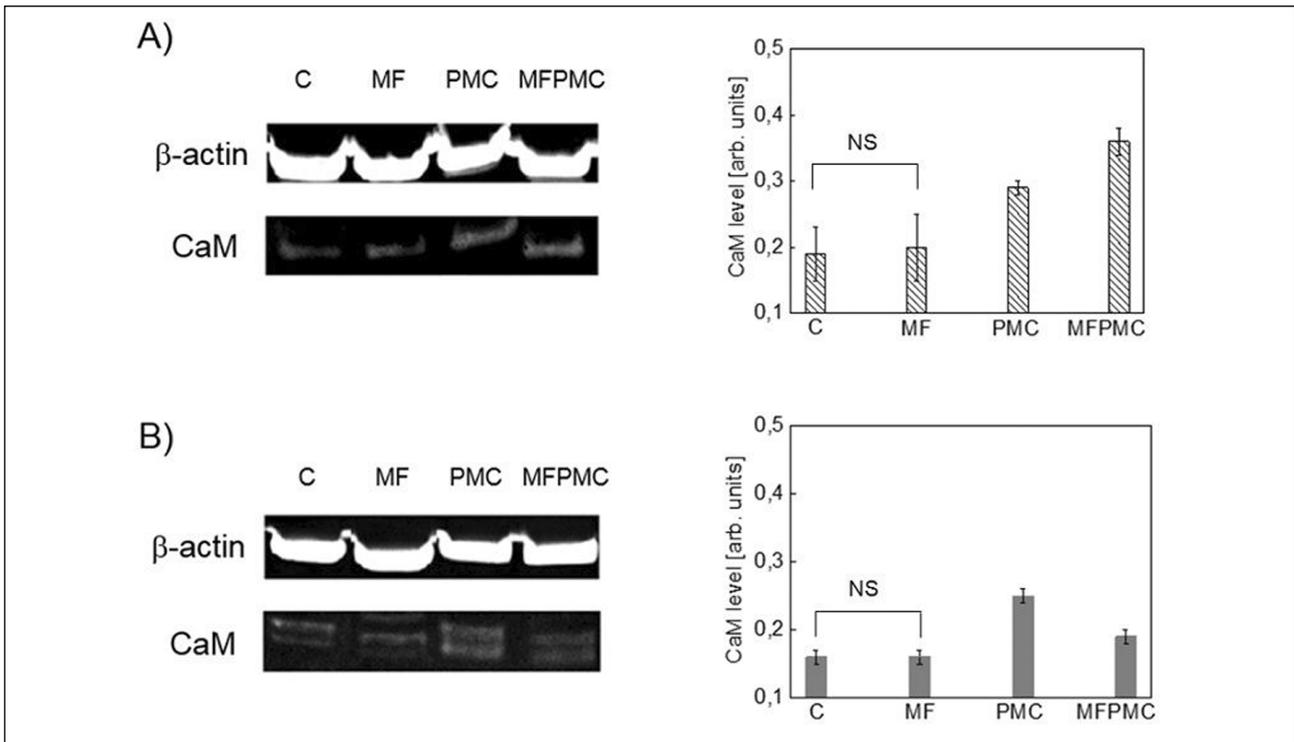
Fig. 2. Western blot of CaM protein products in U937 cells in the absence or in the presence of MF and/or PMC. (A) refers to the ACDCMF (6 mT + 6.5 $_{rms}$ mT; 35 Hz) and/or PMC conditions; (B) refers to U937 cells stimulated with PMC and/or PEMF (45 ± 5 mT; 50 Hz). Protein levels were normalized to β-actin signals for lanes. Data are expressed as mean ± S.D. All differences are statistically significant P < 0.05 (expect for comparison between C and MF samples). C, control sample; PMC, U937 cells treated with puromycin; MF, MF exposed cells; MFPMC, MF and PMC stimulated cells; NS, not statistically significant (P > 0.05). Bands from representative experiment out of three independent, each done in duplicate are shown.
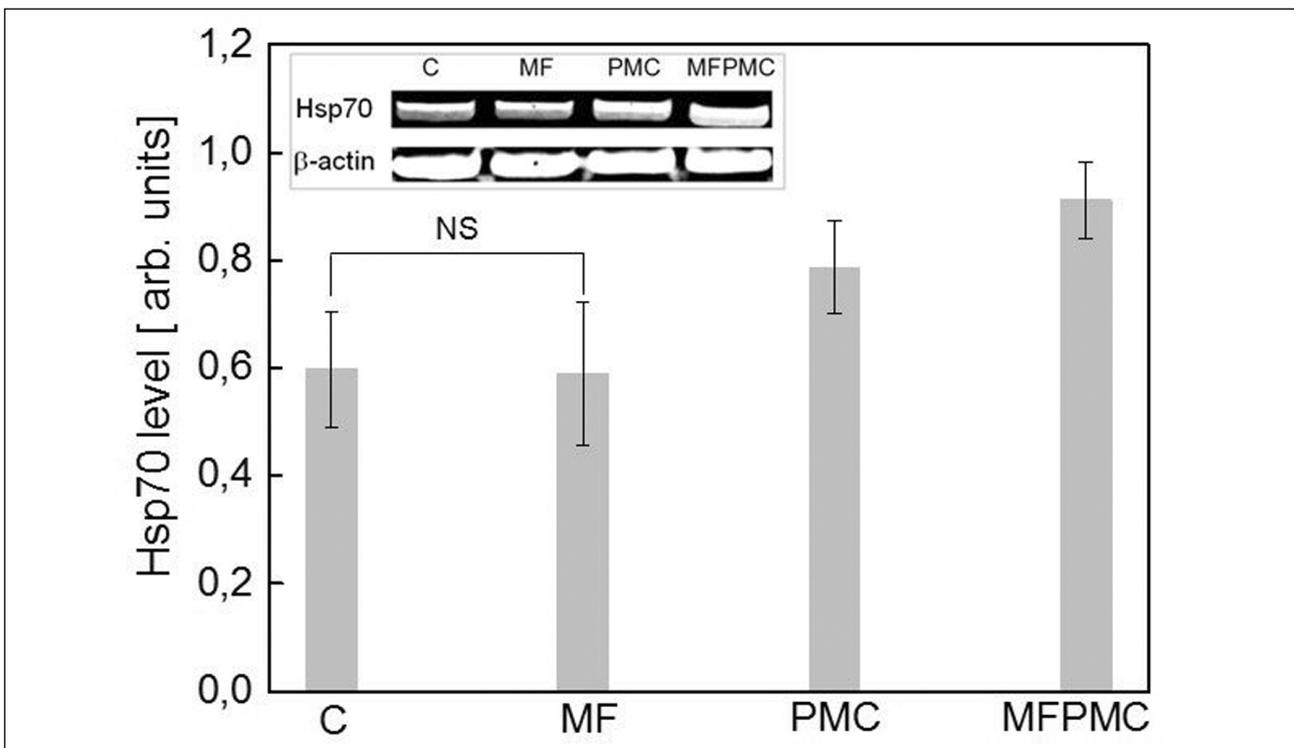
Fig. 3. Western blot of Hsp70 protein products in U937 cells in the absence or in the presence of ACDCMF (6 mT + 6.5 $_{rms}$ mT; 35 Hz) and/or PMC. Protein levels were normalized to β-actin signals for lanes. Data are expressed as mean ± S.D. All differences are statistically significant P < 0.05 (expect for comparison between C and MF samples). C, control sample; PMC, U937 cells treated with puromycin; MF, cells exposed to magnetic field; MFPMC, cells stimulated with magnetic field and PMC; NS, not statistically significant (P > 0.05). Bands from representative experiment out of three independent, each done in triplicate are shown.

Western blot analysis of Hsp70 protein products in U937 cells in the absence and in the presence of ACDCMF and/or PMC (*Fig. 3*) has shown, that the Hsp70 level was strictly correlated with the amount of CaM (*Fig. 2A*) in the relevant samples. Examination of immunoblots developed with anti-Hsp70 antibody have shown, that amount of Hsp70 protein in control- and only MF-treated samples was preserved without significant changes. However, Hsp70 protein content has been increased, in both, PMC-treated sample and in sample simultaneously stimulated with magnetic field and PMC, compared to control one. The highest content of Hsp70 and CaM proteins has been related to the lowest U937 cell viability under action of applied ACDCMF and PMC stress factors.

Therefore, calmodulin orchestrates action of various proteins involved in cell death- or cell survival signalling, it is likely, that observed modulations of the levels of CaM and Hsp70 proteins might influence cell functioning. The role of CaM-dependent signalling pathways in maintaining of cell homeostasis was confirmed in (24). The observed relation between intracellular levels of CaM and Hsp70 proteins and cell fate under applied inconvenient conditions, indicates the direction of further studies concerning MFs' influence on leukemic cells survival.

DISCUSSION

Foregoing findings concerning action of MF alone on cell function are consistent with conclusions presented by some other groups (29, 33, 34). However, there are also reports presenting contrary results (2, 35, 36). Existing controversies reveal dependence between caused bio-effects, cell types and exposure conditions (8). The use of a known cytotoxic agent (PMC) reduced population of viable cells in dose-dependent manner, which stays in agreement with other studies (20, 28). Puromycin is the apoptotic inducer that plays a multiple role in cell *via* inhibition of translation in protein synthesis process or *via* acting as ER Ca^{2+} leak channel opener and an ER stressor (22, 26). This multitasking role of PMC makes it a good candidate for performed studies. Combined action of MF with PMC influenced cell viability differently for two types of MF exposure systems, and these effects cannot be explain through MF's interaction with PMC itself. There is no evidence for changes in PMC or other apoptotic agents transport into the cell due to similar conditions of MF- exposure (magnetic flux density and time of MF's application) (37-39, 41). Thus, the synergic action of MF and PMC might rather determine internal changes in the studied system, which finally could trigger signal cascades leading to cell survival or cell death. Indeed, it has been shown, that changes in cell functioning might be the result of the modulation of apoptosis-related genes expression, which regulate transduction of multiple signal pathways (39). In the study of Kaszuba-Zwoinska *et al.* (40), the variations in content of Bcl-2 family gene members and AIF gene under PEMF and/or PMC stimulation in MonoMac6 cells were presented. The authors have observed, that PEMF interaction elevated Bcl-2 mRNA in PMC treated cells, but diminished mRNA of pro-apoptotic Bax gene. Thus, used PEMF fulfilled protective role against of cytotoxic agent- induced cell death. The synergic action of magnetic fields and chemical stressors in cells are also supported by findings of other authors. In the study of Keczan *et al.* (42), the authors have focused on PEMF and/or tunicamycin- induced endoplasmic stress in three different cell lines. They have observed changes in the level of ER stress- and apoptosis markers in dependence on cell types and applied stressor(s). For human liver carcinoma (HepG2) cells, exposure to PEMF has reduced the elevated level of ER chaperons and apoptosis marker induced by tunicamycin, thus improving cell viability.

The similar protective effect for PEMF and PMC was observed in the studied here U937 cells. The discrepancy between results for ACDCMF or PEMF are most likely related to the parameters of used MF itself. It has been indicated before, that measurable bio-effects are dependent not only on magnetic flux density and/or frequency, but also on combinations of static- and alternating MF components, which might determine occurrence of resonant effects (8). Although, the physical mechanism of MF action has not yet been fully elucidated and is still unclear (6, 7, 10, 15, 13, 25). There is still lack of data concerning the linkage: physical factor (MF) - intracellular target of MF - measured bio-effects (cell response). The problem is all the more urgent, that one can notice rising exposure to magnetic fields and observed health problems in populations living close to magnetic fields sources (43, 44). In addition, MF are already used in medicine for treatment of some disorders, despite of lack of well-established theoretical model of their action on cells (45, 46).

However, as the calcium homeostasis is crucial for normal cell functioning, the processes of calcium mobilization and Ca^{2+}-dependent signalling cascades are considered as a very likely 'cellular targets' of MF (15, 26, 30). The great role in calcium homeostasis is fulfilled by the heterogeneous group of Ca^{2+}-binding proteins (CBP) capable of decoding very small changes in calcium intracellular concentration. For instance, calmodulin is an ubiquitous, versatile Ca^{2+}- binding protein, which triggers multiple cellular processes including programmed cell death (47-49). To the group of well-characterized calmodulin target proteins belongs protein kinases, calcineurin (CaN), nitric oxide synthase (NOS), inositol 3-kinase, Ca^{2+}-ATPase, IP_3 receptors (IP_3R), cytoskeleton proteins, receptors and ion channels (e.g. SOC, endoplasmic reticulum-translocon (TLC)), heat shock proteins (50-57). The concentration of CaM in resting cells is kept low, but could be down- or upregulated in accordance with modulation of intracellular Ca^{2+} level as a response to chemical carcinogens, ionizing and non-ionizing radiation or phase of the cell cycle (58-60).

Thus, we have examined PMC and MFs (as the external stressors) on production of CaM and Hsp70 proteins in the model cell line. The Hsp70 protein is one of Ca^{2+}-dependent-CaM-binding proteins crucial for cell survival. The modulation of the level of heat shock proteins can affect apoptosis process differently in various cell types. For instance, in the study of Leja-Szpak *et al.* (61) and related one (62), it was shown that kynuramines and melatonin stimulate production of heat shock proteins in pancreatic carcinoma cells (PANC-1), which resulted in the interruption of the intrinisic apoptotic pathway. In the study of Galea-Lauri *et al.* (63) for U937 cells, it has been verfied that heat shock proteins are implicated in the regulation of forms of cell injury that lead to programmed cell death. An excess of Hsp90 was associated with increased apoptosis when the cells were treated with a combination of TNF-alpha and cycloheximide, and what is more, new synthesis of Hsp72 did not protect against apoptosis. Chant *et al.* (64) have observed that susceptibility of acute myeloid leukaemia (AML) cells to apoptosis correlated with intracellular expression of Hsp70 and was associated with the presence of p53 and low levels of expression of Bcl-2.

Herein, the level of CaM and Hsp70 proteins was changed for particular combination of external stress factors, as well as cell viability. It indicates that CaM and CaM-dependent signalling molecules might posses a role in intracellular mechanism(s) responsible for cell response to used stimulation. Due to the fact that expression of CaM/Ca^{2+}-dependent-CaM-binding proteins and cell survival are modulated with the changes in intracellular Ca^{2+} ions concentration, it is likely, that applied stress conditions have triggered calcium ions fluctuations in examined cells. Indeed, the increase in the Ca^{2+} leak through TLC under PMC treatment, can lead to cytosolic calcium ions elevation and to initiation of a number of cellular responses, for instance,

upregulation of CaM (58, 59) and cell death (22, 28, 59, 65-67). Thus, our results seem to be consistent with findings indicating that changes in intracellular Ca^{2+} concentration can entail changes in CaM level, in consequence modulating Ca^{2+}-CaM- dependent signalling pathways and cell functioning. For instance, according with the study of Dowd *et al.* (68), calmodulin gene expression was upregulated in WEHI7.2 lymphocytes undergoing glucocorticoid-mediated apoptosis. Authors have reported induction of CaM gene expression by dexamethasone, and other data supporting the putative role of CaM in apoptosis. The authors also claimed that CaM may play several key roles in cell death process, what should be intensively verified. In the study of Yu *et al.* (69), the mechanism of pancreatic beta cell loss in transgenic mice with elevated levels of calmodulin was presented. The authors speculate that overexpression of calmodulin sensitizes the beta cells to commit apoptosis *via* Ca^{2+}-dependent activation of nitric oxide synthase, which suggests that the cell death process is Ca^{2+}/CaM- dependent. Devireddy and Green (70) studies on IL-2-dependent T cells have revealed the role of a pro-apoptotic factor, RC3, whose ability to induce programmed cell death is related to its role in modulating intracellular CaM and/or Ca^{2+} levels. In the study of Pilla *et al.* (30), Ca^{2+} binding to CaM driven by increase in cytosolic Ca^{2+} concentration in response to chemical and/or physical insults (e.g. MF), activates the synthesis of the signalling molecule NO from L-arginine, which further triggers development of signalling cascades crucial for cell survival or death.

Also the obtained here relation between enhanced cell death in cells under stress conditions and the intracellular level of heat shock protein (Hsp70), stays in agreement with other data for leukemic cells. The observed in this study changes in both proteins (CaM and Hsp70) content and in U937 cell viability upon exposure to magnetic fields acting alone or when combined with cytotoxic agent, may be useful to indicate intracellular processes, which are likely activated by applied MFs.

However, in contrast to the general paradigm, assuming that rise of cytosolic Ca^{2+} level evoked by external stressor(s) is characteristic for cells committed to apoptosis, other studies have reported that elevation of calcium ions in cytosol can exert either a pro- or an anti-apoptotic effect, and also CaM may play a double role (71-73). Both types of effects can occur in dependence on cell types, conditions of cell system, dynamics and spatial range of calcium signal itself. For instance, Ca^{2+}-calmodulin-dependent activity of calcineurin, was shown to promote apoptosis through enhancing binding of dephosphorylated pro-apoptotic molecule Bad with Bcl-xL protein (74). In turn, PKC isoforms can play double role (75).

In this context, the detailed explanation of our data needs further studies concerning various intensities of MF, influence of calcium channel blockers, and several downstream enzymes under the control of calmodulin. Also cooperative involvement of other mechanisms like stress proteins-mediated signalling pathways cannot be excluded. Thus far, we can assume, that observed differences in cell viability might be related to modulation of CaM and Hsp70 proteins concentrations as a cell response to varied stress conditions, but the detailed bio-mechanism requires more research.

Conclusions

The exposure to MFs combined with PMC affects U937 cell viability and level of both CaM and Hsp70 proteins. Observed changes are dependent on parameters of applied MF, and are not consequence of direct interaction between the field and the apoptosis inducer (PMC). From our data, it could be hypothesized that occurred modulation of cell viability might be result of Ca^{2+}- dependent signalling perturbation, as calmodulin acts as the primary intracellular calcium sensor. Calmodulin regulates numerous proteins in response to changes in the intracellular calcium content (e.g. Hsp70), thus triggering multitude of cell signalling cascades which eventually might lead to cell survival or to cell death. Nonetheless, the detailed mechanism underlies the interaction between used MFs and model cell line is still not recognized and needs further studies. Understanding the intracellular bio-effects evoked by MFs act in synergy with chemical agents is crucial to develop new strategies for noninvasive cancer therapy or for protection against rising exposure to these stress factors.

Abbreviations: 7-AAD, 7-amino-actinomycin D; ACMF, alternating magnetic field; AML, acute myeloid leukaemia; AnV-APC, annexin V allophycocyanin labeled; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+} concentration; CaM, calmodulin; CaN, calcineurin; CBP, calcium binding proteins; DCMF, static magnetic field; ER, endoplasmic reticulum; f, frequency; HepG2, human liver carcinoma; IL-2, interleukin-2; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptors; NBT, nitro blue tetrazolium chloride; NOS, nitric oxide synthase; PANC-1, pancreatic carcinoma cells; PEMF, pulsed electromagnetic field; PKC, protein kinase C; PMC, puromycin; rms, root mean square; S, cell viability coefficient; SOC, store-gated channels; TLC, translocon; U937, human leukemia cell line; VP16, topoisomerase II inhibitor etoposide; β-actin, actin isoform.

Conflict of interests: None declared.

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