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ARTICLE

Effects of 6-epi-ophiobolin A on mechanical and physiological parameters of cardiomyocytes and on the reorganization and amounts of their kv4.x ion channels

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ABSTRACT Ophiobolins are important members of the family of phytotoxic metabolites, and they possess antitumor, antibacterial, antifungal activities. At the same time, the knowledge on the effect of ophiobolins on ion channels is very limited. The voltage-dependent transient outward currents (I_{to}) are determined by kv4.2 and kv4.3 ion channels. Increasing evidence points to a role of HMGB1 and synapse-associated protein 97 (Sap97) as underlying factors related to inflammation. The Sap97 associates with kv4.x type channels in the complex and modulating their kinetic properties. Our hypothesis was that Sap97 mainly localizes in the intercalated discs of the cardiac muscle, therefore the altered expression of ion channels is presumably involved in the inhibition of normal mechanical parameters and physiological function of transient outward current (I_{to}). Our approach was to use purified 6-epi-ophiobolin A as effective molecular tools in the investigation of ion channels on cardiomyocytes. In this work, we have investigated the mechanical factors by atomic force microscope (AFM) technique and the surface expression of kv4 ion channels on the cardiomyocytes using confocal microscopy after immunofluorescence labeling. AFM study revealed that the elasticity of the cell surface, the Young's modulus had been moderately changed, as well as the cell volume and the heights of the cells in the presence of low concentration of 6-epi-ophiobolin A. After this treatment the Sap97 binding to kv4.3 or kv4.2 channels and distribution of their complexes are also changed in the membrane accompanied by altered physiological behavior of cardiomyocytes as compared to control. A growing number of researches using these new reductionist models of inflammation on cardiomyocytes, demonstrate a role of Sap97 in specific ion channel stability important for cardiac functions. These results suggest that Sap97 deactivation or reduction can lead (directly or indirectly) to changes in the functional cell surface expression of kv4.x channels with mechanical parameters, with biophysical and biochemical properties of cardiac I_{to} current.
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KEY WORDS

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transient outward current

Introduction

Nowadays, the effects of the secondary metabolites come into sight since they can influence the electrolyte balance in humans or animals, but their biological consequence is not well known. The Ca^{2+}-independent voltage-gated transient outward current (I_{to}) is important for maintenance of ionic currents in the first period of the repolarization phase of action potential in cardiomyocytes (Heufbach et al. 2003). Transient outward current is evolved mainly by kv4.2 and kv4.3 in human cardiomyocytes, and acts in the first period of repolarization phase of the action potential (AP) (Liu et al. 2015).

An essential characteristic that differentiates the voltage-gated potassium (Kv) channels from both the cardiac sodium (Nav) and calcium (Cav) channels is the variation in terms

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of electrophysiology and function of the K^+ channels (Nerbonne and Kass 2005). Two classes of repolarizing cardiac Kv currents can be mainly distinguished; the transient outward potassium current (I_{to}), which plays an important role in phase I of the AP, and the delayed, outwardly rectifying potassium current (I_K) (Wettwer et al. 1994; Nattel et al. 2007). At membrane potentials, above 30 mV both of these types are activated (Barry and Nerbonne 1996). Within these two major groups, there are further subdivisions of the I_{to} and I_K depending on various time- and voltage properties. Due to the different densities and biophysical qualities of these channels throughout the various regions of the heart, various waveforms of APs could be observed. For example, regional differential expressions of transient Kv currents were found between in the left ventricular endocardium and epicardium. The first region showed lower I_{to} density and very slow recovery, while in the second region, higher I_{to} density and fast recovery were observed (Brahmajothi et al. 1999). This variation of the distribution and kinetics of the I_{to} must be an important factor in the regulation of the physical cardiac rhythm and an even more significant factor in the pathological heart (Nerbonne and Kass 2005; Wettwer et al. 2013).

Furthermore, the physiologically active kv4.x channels are associated with several ancillary subunits such as β-subunits (kvβ1 and kvβ2), Kv channel-interacting proteins (KCHIP) acting as chaperons (Akar et al. 2004). KCHIPs regulate the surface expression of channels and determine the electrophysiological properties. The kv4.x ion channel function is dependent of the Kv channel complex specific subcellular localization and trafficking mechanisms (Kääb et al. 1998; Takeutchi et al. 2000; Chang-Liao et al. 2015). Kv4.x channel complex with kv4.3 pore-forming α-subunits, and auxiliary subunits KCHIPs, DPP6 and DPP10 were found as components of kv4.x channel complex modulating their currents in brain and in the myocytes as well as in other tissues (Radicke et al. 2005; Maffie and Rudy 2008; Cotella et al. 2010, 2012; Turnow et al. 2015). These genes with others for ion channel proteins are involved in the consequences of cardiomyopathy such as in the arrhythmogenesis of coronary artery disease and ventricular arrhythmias with sudden cardiac death (El-Haou et al. 2009; Nattel et al. 2007; Gaborit et al. 2009). Kv4.x α-subunits associate with synapse-associated protein 97 (Sap97) kinase (El-Haou et al. 2009; Fourie et al. 2014) and with high-mobility group box-1 (HMGB1) non-histone chromatin-associated protein, which modulates the normal physiological function of ion channels (Wang et al. 1999; Au et al. 2000; Yang et al. 2004, 2005; Liu et al. 2010; Kang et al. 2014). The Sap97 anchors the kv4.x ion channels and regulates the subcellular translocation and localization of the channels (Fourie et al. 2014). Kv4.x has been proven to concentrate in the sarcolemma of the ventricular myocytes at the intercalated disk region, which connects myocytes (Milstein et al. 2012; Liu et al. 2015).

HMGB1 performs diverse intracellular and extracellular functions in health and disease. In the nucleus, it regulates chromatin architecture, transcription, replication and repair processes. In addition, HMGB1 secreted by macrophages and necrotic cells also performs various intracellular and extracellular activities as an alarming danger signal during injury and diseases such as inflammation, arthritis, multiple human diseases including infectious diseases, ischemia, immune disorders, neurodegenerative diseases, metabolic disorders and cancer (Hock et al. 2007; Lei 2013; Kang et al. 2014, Roy et al. 2016). HMGB1 is the prototypic damage associated molecular pattern molecule (DAMP). HMGB1 is in conjunction with other factors, thus has cytokine, chemokine, and growth factor activity, orchestrating the inflammatory and immune response. HMGB1 location and function largely depend on redox states (Qiu et al. 2011).

The objective of Atomic Force Microscope (AFM) is the micro- and nanometer scale characterization of different biological samples in different fields such as peptides and oligonucleotides (Butt and Jaschke 1995; Bálint et al. 2007), photosynthetic reaction centers, membrane proteins (Bálint et al. 2007, Caloni et al. 2012) and the investigation of living cell's surface and their responses to different extracellular stimuli also available (Bálint et al. 2007; Wilhelm et al. 2007; Harmati et al. 2016). The AFM's possibility to work in liquid environment and especially the design of the MFP 3D type AFM made possible to study living cells *in situ*, in their physiological circumstances.

Most of the microbial secondary metabolites have substantial effects on humans and animals, but the background of their action is usually unclarified. The molecular mechanisms associated with these bioactive metabolites underlying stress conditions of I_{to} are still largely unknown.

Ophiobolins are a group of naturally occurring sesterterpene-type secondary metabolites with a specific tricyclic ring system (5-8-5) named as A-, B- and C-ring, respectively. They are mainly produced by filamentous fungi belonging to the genus *Bipolaris*, however, several ophiobolin analogues have been reported from other genera, such as *Drechslera*, *Cephalosporium*, *Ulocladium* and *Aspergillus* (Wei et al. 2004; Wang et al. 2013). Until now, 50 different ophiobolin analogues have been reported and characterized, 18 of them in the last five years. Interestingly, most of the ophiobolins were isolated from terrestrial microorganisms; however, many of the recently discovered ophiobolin analogues were isolated from marine microorganisms, which have become preferable sources of new bioactive compounds in the last decade (Wei et al. 2004; Wang et al. 2013; Bencsik et al. 2014). The best-known member of the ophiobolin family, the ophiobolin A exhibits a broad spectrum of biological activities such as phytotoxic, calmodulin antagonist, antimicrobial, antimalarial, nematocid or anticancer effects. Nowadays, the effect of ophiobolin A was demonstrated on big conductance

Ca-channels (BKCa): it decreased the BKCa channel activity (Bury et al. 2013).

In this study, we investigated whether the 6-epi-ophiobolin A (6EOPA) is able to modulate the distribution of kv4.x ion channels or there is any relation of the treatment to inflammation or has any effect to the mechanical characters of the cardiomyocytes. Furthermore, the molecular details about the association between the kv4.x channels and Sap97 subunits were also investigated. Our approach was to compare the localization and distribution of kv4.x channels and the anchoring protein Sap97 in the cardiomyopathy. For this reason, the localization and distribution of kv4.x channels and their modulators like Sap97 were recorded in the presence of derivatives of 6EOPA in cardiomyocytes under external stress caused by fungal metabolites. For the assays, the heart cell line of H9c2 rat was used as a tool to localize kv4.3 ion channels with correlated confocal microscopic methods with immunohistochemistry. Furthermore, understanding the cell model the AFM technique was applied to measure "stiffness" on the surface of cardiomyocytes showed the alteration of the cell surface, which could lead to change the pharmacological properties of ionic currents in cardiac cells.

Materials and Methods

Cell cultures of rat cardiomyocytes and cell treatment

Rat cardiomyocyte H9c2 cell line was a gift from E. Duda (HAAS, BRC, Szeged, Hungary), which were grown in Dulbecco's modified Eagle's medium (PAA, Austria) supplemented with 10% foetal bovine serum (FBS; PAA, Austria), 100 U/ml penicillin and 100 mg/ml streptomycin (Pen-Strep; Lonza, Hungary) in standard conditions (37 °C, 5% CO_2). The cells were grown in round glass coverslip, until 70% density both for immunofluorescence and AFM studies. For the treatment, 6EOPA was used at 2.8 x 10^{-6} mg/ml final concentration added to the medium. After 24 h, the cells were attached to the coverslip and stored at -20 °C until use both for fluorescence immunocytochemistry and AFM investigations.

To test the cytokine effects, antibody of anti-HMGB1 was used (GST-HMGB1 61 ng/ml in the medium, it marked as HMGB1), where the concentration was considered according to the literature (Liu et al. 2010; Szénási et al. 2013).

Immunofluorescent study on cardiomyocytes

For the immunofluorescent staining, the cells were blocked with 2% BSA in calcium-free phosphate-buffered saline (PBS) and incubated for 2 h at room temperature either with rabbit anti-kv4.2 (diluted 1:100; Alomone, Israel) or anti-kv4.3 (diluted 1:100; Sigma-Aldrich, Hungary) and mouse anti-Sap97 antibody (diluted 1:300; LifeSpan BioSciences, USA). Cells were then washed and incubated with Alexa 488-labeled anti-rabbit IgG (diluted 1:400, Molecular Probes) plus cy3-conjugated anti-mouse IgG (diluted: 1:400; Jackson Immunoresearch, USA) for 1 h at room temperature. Nuclei were stained with 1 μg/ml Hoechst 33258 (Sigma-Aldrich, Hungary) in PBS for 10 min. After rinsing, sections were mounted with fluorescent mounting medium (Agilent, USA), viewed and photographed with a confocal laser scanning microscope using an Olympus Fluoview FV1000 (Olympus, Germany).

Purification of 6-epi-ophiobolin A

The purification and separation of 6EOPA was performed by semi-preparative HPLC method from the ferment broth of *Bipolaris oryze* (SZMC 13003) based on earlier report (Bencsik et al. 2014). The identification of the 6EOPA compound (purity >96%) was carried out using LC-MS analysis and NMR examinations.

Measuring nanomechanical parameters of cells by atomic force microscope technique

The surface morphology, topography, and mechanical properties of 6EOPA treated cells were evaluated by using an AFM coupled with an inverted optical microscope (Axiovert 200, Zeiss, Hungary). Briefly, the combined inverted optical stage helped precise lateral positioning of the AFM tip over the region of cell tissues of target materials. The measurements were carried out with an Asylum MFP-3D head and Molecular Force Probe 3D controller (Asylum Research, USA) in force mode. The driver program MFP-3D Xp (ver. 13.17.101) was written in IGOR Pro software (ver. 6.37, Wavemetrics, USA). The silicon nitride cantilevers (Bio-Lever BL-RC150VB-C1, Olympus, Japan) with the nominal spring constant of 30 pN/nm in air were used for the experiments came from the same wafer. Calibration of the real spring constant of each individual cantilever was performed by thermal fluctuation technique (Butt and Jaschke 1995; Proksch et al. 2004) and the Sader method (Sader et al. 2012). The scan size was adjusted to 80 × 80 μm^2 to accommodate typical surface features, while maintaining a high resolution. The Young´s modulus was subsequently determined by using the Hertz´s contact model. For calculation of material stiffness, the Poisson's ratio was assumed 0.5, the ramp size was 1 μm, and the loading speed was 1 μm/s. A series of indentation forces (0.5-10 nN) were tested to calibrate, whether the indentation depth was in the proper range between 20-200 nm to prevent substrate surface defects and Hertz´s model limitation. The Young's modulus was resolved using the DMT's contact model.

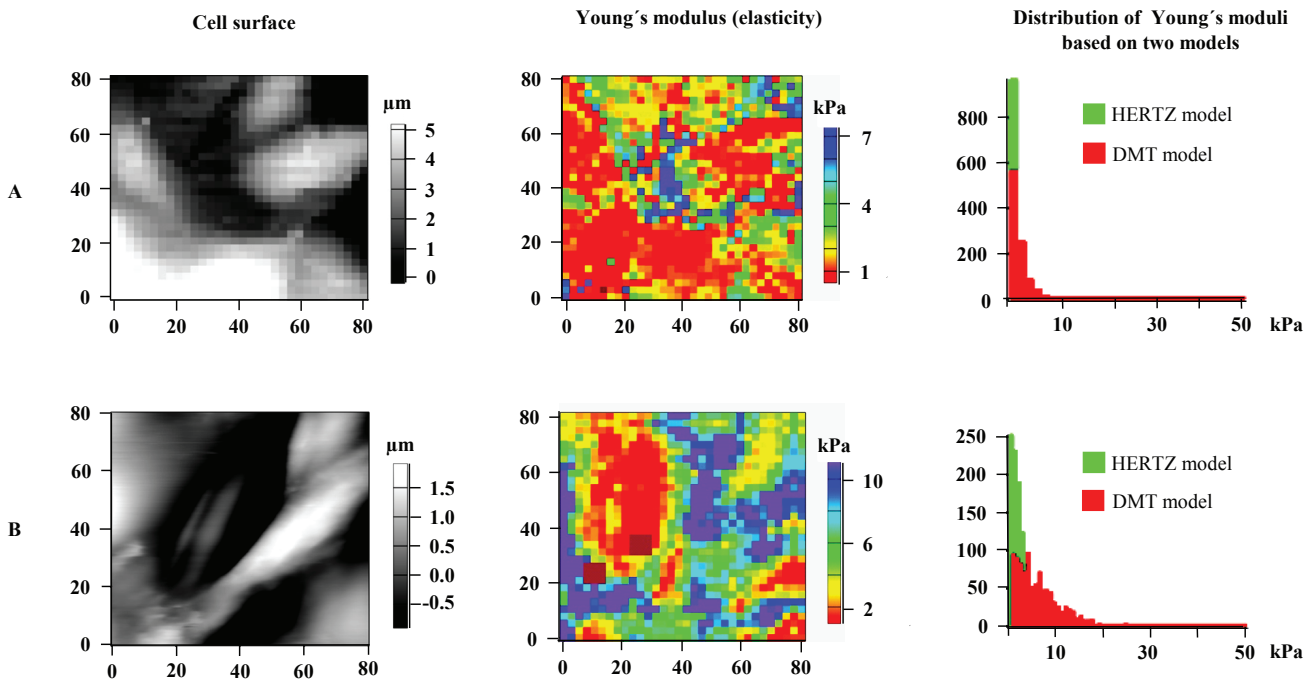

Figure 1. Effect of 6EOPA on the nanomechanical parameters of the heart cells surfaces measured with atomic force microscope technique. Young's modulus characterizes the elasticity of the cells, which is moderately changed in the presence of low ophiobolin concentration. 6EOPA:6-epi-ophiobolin A; A: Control cells, B: 6EOPA treated cells..

Results

The membrane surface tension was investigated by AFM method and the distribution of kv4.2 and kv4.3 ion channels in the membrane of H9c2 heart cell line was investigated using immunofluorescence labeling.

Effect of 6-epi-ophiobolin A on mechanical parameters of cardiomyocytes

The effect of 6EOPA treatment on the nanomechanical properties of membrane surface of rat cardiomyocytes was measured with AFM method (Fig. 1). The heights of cells, the stiffness of the membrane and the elasticity were investigated. Elasticity was characterized with Young's modulus of the cell surface, which was changed moderately after adding low concentration 6EOPA.

Heights of cardiomyocytes were calculated by two models, both were demonstrated the average heights of cells in microscope. The height of cardiomyocytes was altered mildly and the values of Hertz and DMT model for plasma membrane (PM) are shown in Figure 1. Both micrographs and the calculated Young's modulus figures indicated the different topology of surface for healthy and treated myocytes. This was confirmed by kPa values in Hertz and DMT model, where these values were increased by about 50% for each treated cell compared to healthy controls presenting with representative figure.

PM of cardiomyocytes was characterized by mechanical parameters in the presence of 6EOPA. Young's modulus characterizes the elasticity of the cell surface, which is moderately changed in the presence of low ophiobolin concentration (Fig. 1).

Environmental effects on kv4.2 and kv4.3 ion channels

Specifically labeled kv4.2 and kv4.3 channels were studied on adult rat cardiomyocytes in healthy cell line and 6EOPA treated cells. These experiments were performed parallel with AFM measuring with low concentration of 6EOPA.

Kv4.2 and Sap97 channel proteins were expressed abundantly in the PM of healthy cells and they colocalized at the lateral side of PM and around nuclear membrane (Fig. 2). Kv4.2 channels were labeled fainter at the lateral side. Decreased number of kv4.2 channels was detected in the PM, also the cytoplasmic area in treated cells with HMGB1

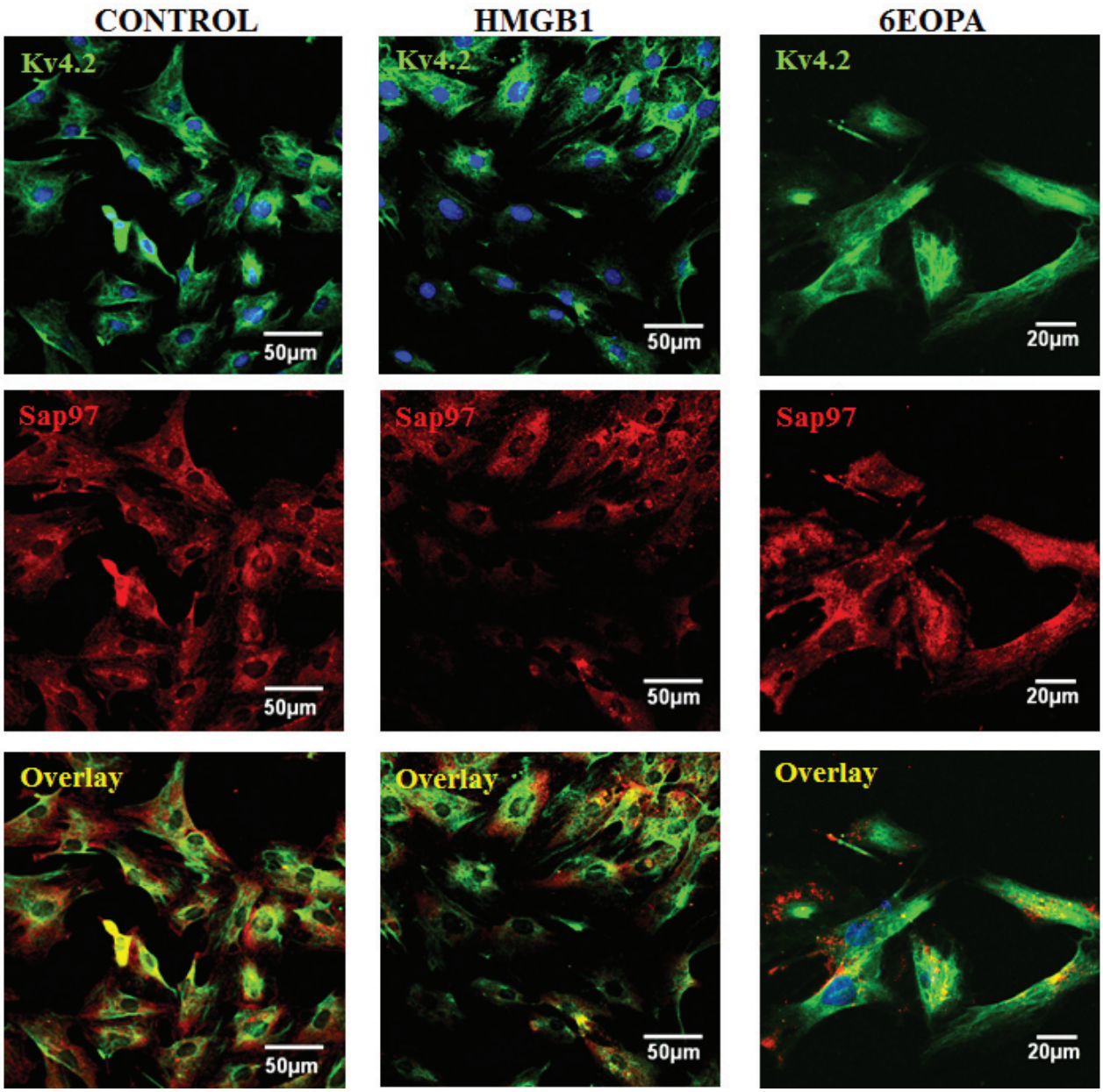

Figure 2. Immunofluorescent staining of kv4.2 ion channel after 6EOPA or HMGB1 treatments in rat heart cells. Kv4.2 ion channel colocalizes with Sap97 anchoring protein in the plasma membrane and in cytoplasm of cardiomyocytes. This association and distribution were altered after 6 EOPA or HMGB1 treatment in rat heart cells. 6 EOPA: 6-epi-ophiobolin A; HMGB1: high mobility binding protein 1; Sap97: Synaptic associated protein 97.

or 6EOPA. The amount of Sap97 protein was decreased and colocalization could not be detected with kv4.2 protein in the presence of HMGB1, while some colocalization was visible after 6EOPA treatment in few cells (Fig. 2).

To examine the 6EOPA or HMGB1 effect on kv4.3 channels, the same expanded cell cultures were used. Kv4.3 ion channels have strong labeling on the PM and on the membrane of the nucleus colocalized with Sap97 proteins (Fig. 3). Following treatment with 6EOPA, less amount of kv4.2 and kv4.3 channels were detected around the nucleus (Figs. 2, 3). The labeling was shown little faint sign in the PM, too.

The same effects were detected on kv4.2 and kv4.3 chan-

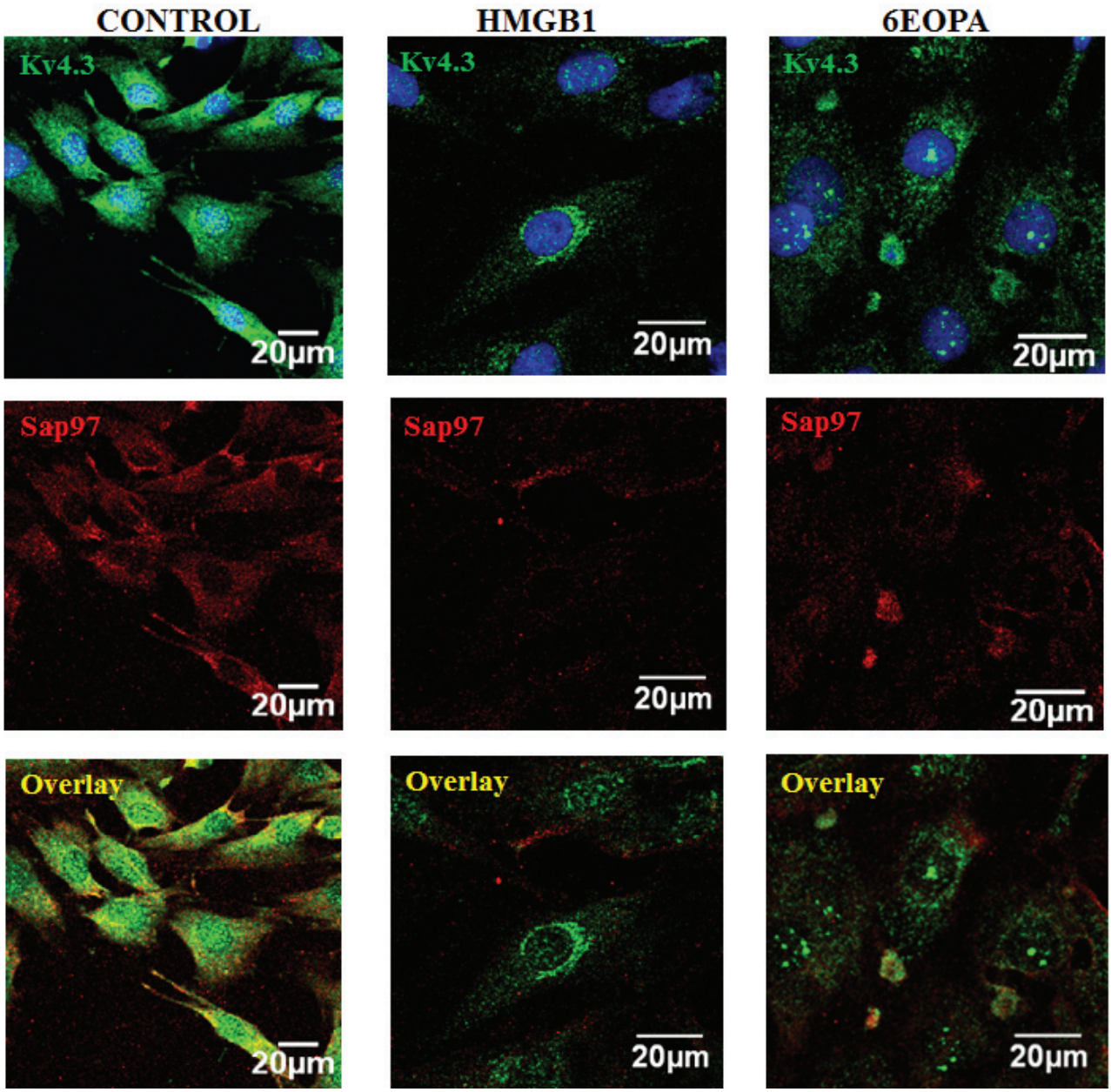

Figure 3. Immunofluorescent staining of kv4.3 ion channel after 6EOPA or HMGB1 treatments in rat heart cells. Kv4.3 ion channel and Sap97 protein were colocalized partially in the presence of 6EOPA or HMGB1 in cardiomyocytes. 6EOPA, 6-epi-ophiobolin A; HMGB1, high mobility binding protein 1; Sap97: Synaptic associated protein 97.

nels in the treatment of HMGB1 or 6EOPA. Effect of low concentration of 6EOPA was exerted on kv4.2 and kv4.3 channels.

Colocalization of Sap97 with kv4.2 after HMGB1 or 6EOPA treatment decreased or could not be detected in rat heart cells.

We were interested in the alteration of expressed kv4.x channels and distribution with Sap97 in cardiomyocytes after external stress and wether 6EOPA influences this colocalization. The high intensity shows the abundant kv4.3 in the control rat cardiomyocytes overlapping with Sap97. It changed upon adding stress factors HMGB1 as a cytokine and 6EOPA, respectively. The presence of HMGB1 or 6EOPA exerted the same down-regulation on the kv4.3 channel complexes.

Discussion

To address the importance of potassium channels, we investigated the effects of the 6EOPA as external stress factor and HMGB1 as a cytokine on kv4.x channels in heart cells. Novel results are: (1) The kv4.3 ion channels localized mainly around the PM and on the membrane surface as well as on the nuclear membrane of rat cardiomyocytes. (2) Distribution and amounts of kv4.2 and kv4.3 ion channel complexes changed in the presence of 6EOPA and HMGB1 in cardiomyocytes. (3) Immunostaining revealed that Sap97 co-localization with kv4.3 ion channel α-subunits altered in the presence of 6EOPA at the sarcolemma and may also change in other subcellular organs compared to normal healthy cardiomyocytes. AFM measurements were used to obtain new information from cell surface alteration caused by OPA derivatives on the cardiomyocytes. AFM makes possible to investigate membrane rigidity caused by severe heart or muscle diseases, leading to reversible and irreversible changes on the cardiomyocytes. The results of AFM had an opportunity adapting methods, directly on living cells, which is the most human-like cardiomyocyte model. The examination of this cell model may help to find an appropriate drug to develop new methods for testing effective treatments in diseases.

AFM study showed that the Young's modulus and the mechanical parameters are altered on the cell surface after one day in the presence of 6EOPA. In this investigation, the kPa values in Hertz and DMT model indicate the altered nanomechanical parameters for mild effects on or in the PM. In adult cardiomyocytes, increased heights were measured suggesting the different structure of PM from healthy cells, which could be even reversible due to the used low concentration of 6EOPA tolerating the applied circumstances. These data may not be applicable for altered parameters of PM and the other biophysical and biochemical background in acute treatment, only when the latest method, the combination of AFM with patch-clamp study could be adapted (Ossola et al. 2015; Chen et al. 2016).

Remodeling of ion channel expression and function may be the result of modulation of gene transcription, mRNA processing, mRNA translation, protein processing, subunit assembly, membrane transport, assembly into macromolecular complexes and posttranslational regulation. The understanding of this remodeling and the difference between physiological and pathological adaptation will play an important role in the future therapeutic measures against cardiac disease (Rosati and McKinnon 2004). Kv4.x channel protein expression was demonstrated in the intercalated discs in heart tissues (Chen et al. 2016) and supported by earlier studies showing the decreased or increased amount of kv4.x ion channels with western blotting methods in diseased heart cells, which the effects of different drugs (Bányász et al. 2011; Jost et al. 2013; Gómez-Hurtado et al. 2014). In our experiments, down-regulation and redistribution of kv4.2 and kv4.3 ion channels were revealed in the PM after the 6EOPA treatments.

Recently, it was shown that HMGB1 can serve as a proinflammatory marker in tissues, where inflammation caused changes after treatment (Yang et al. 2005, Liu et al. 2010). This highly conserved protein is present in the nuclei and cytoplasm almost in all cell types, but the administration of high amount of HMGB1 to healthy animals causes inflammatory effects including fever, weight loss, anorexia, lung injury, epithelial barrier dysfunction, arthritis and death. The opposite effect, down-regulation of HMGB1 has been shown to inhibit autophagy using lycorine administration in multiple myeloma (Roy et al. 2016).

Our investigations demonstrated the exogenous toxic effects of administrated 6EOPA or high concentration of HMGB1 on kv4.x ion channel complexes anchoring with protein Sap97 in cardiomyocytes. Sap97 protein did not co-localize with kv4.x ion channel α-subunits at the sarcolemma in the presence of 6EOPA or HMGB1 compared to the controls indicating that the anchoring function was disturbed. Sap97 and kv4.x channel subunits co-localize at the sarcolemma of healthy cardiomyocytes, but the distribution of these complexes has changed by treatment of a derivative of 6EOPA. Furthermore, our experiments suggested that HMGB1 treatment may damage also the colocalization of Sap97 with kv4.x channels in all membranes of cell organs as well as in the PM. The results of the present study indicated that the cardiotoxic effects disrupted the ion harmonization in the cells and perhaps the membrane structure was involved in this process.

Earlier the kv4.2 and kv4.3 channels have been detected in the intercalated discs using immunofluorescence methods of healthy cardiomyocytes (Roepke et al. 2008; Foeger et al. 2013; Liu et al. 2015). In our experiments the kv4.2 ion channel expression in healthy rat cardiomyocytes was abundant in the cytoplasm area. The Sap97 anchoring protein, in our experiments, showed the same distribution and co-localization with kv4.2 channels in the cytoplasm of myocytes as well as kv4.3 in healthy myocytes. These results are the first evidence for redistributed kv4.2 as well as the kv4.3 ion channels in the presence of OPA derivative or HMGB1 as exogenous cytokine.

Liu et al. have shown (2010) that the effect of HMGB1 treatment for 24 h: significantly inhibited both the current densities of heterologously expressed kv4.3 and v4.2 in COS-7 cells and the native I_{to} in neonatal rat ventricular myocytes (NRVMs) in a dose-dependent manner. The increased level of HMGB1 in the cytoplasm decreased both the mRNA and protein levels of the α-subunits kv4.2 and kv4.3 channels. We suppose that the 6EOPA treatment changed the heterologous expression of kv4.x channels in PM. Altered kv4.x pattern may be related to the biological activity and

cytotoxicity of some exogenous compound, leads to NF-mediated response under stress effects (Chan et al. 2012; Oeckinghaus et al. 2011).

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