SUBPLASMALEMMAL STRUCTURES OF EXCITABLE AND UNEXCITABLE CELLS IN CLINICAL AND EXPERIMENTAL STUDIES

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Keywords: Transmission electron microscopy, cardiomyocyte subsarcolemma thickness, hepatocytes subcytolemma thickness, inoculation by CCl_4, pulmonary arterial valve stenosis.

The phenomenon of plasmatic membrane (PM) thickness was seldom noted at investigation of cells in different organs. However just presence of such a phenomena was noted, but no valuation of its biological importance was given. In a basis of excitable and unexcitable cells plasmatic membrane thickness changes lays cell submembrane layer thickness alteration. Reversible and irreversible damages of its structures can be a key of cell dysfunction at pathology.

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INTRODUCTION

Plasmatic membrane (PM) is arranged as a biomolecular layer of phospholipids with hydrophilic heads oriented toward extracellular and intracellular compartments. PM is organized into domains which differ in structure and function. Physicochemical properties of PM make it closer to a liquid than to a solid and point to the necessity of formation of inner shell which prevents waning of membrane. Changes in PM thickness as a form of its structure has been mentioned before.[1] PM ability to undergo fast reversible changes of its thickness was observed in the presence of high concentration of Ca^{2+} and phospholipasas.[2] PM local thickness of endotheliocytes of myocardium blood vessels of rat was observed in hypertrophy, while strongly increasing of osmophility and cardiomyocytes sarcolemmas thickness was observed at ischemia and hypoxia.[3,4] It has been shown earlier that during cardiac surgery, 15 minutes after connecting the apparatus of artificial circulation, sarcolemma became more electron-dense, and after another 15 minutes, it was degraded and replaced by large quantity of vesicles.[5] The thicking of cardiomyocytes sarcolemma was also observed in our earlier studies. Increase in PM electron density was shown to occur in cardiomyocytes, endotheliocytes, eritrocytes, connective tissues cells, shvane cells, acsolemmas of vegetative nerve endings of patients with rheumatic and congenital heart defects.[6]

We proposed a hypothesis, that changes in PM thickness on the cell, under different damaging influences, are a universal mechanism of adaptation of its cytoskeleton to the influence. Study of intracellular pressure and the excitable membrane of giant axon suggested, that cytoskeletal structure is involved in the mechanical support of the membrane. Most plausible source of the mechanical response is axsolemma itself.[7] The phenomenon of PM thickness has often been noted during investigation of different cells in different organs. However, just the presence of such a phenomenon has been noted, and no evaluation of its biological importance has been reported.

All data indicate the necessity of an investigation of the phenomenon of changes in the thickness of membrane. Investigations done by our group on the phenomena of changes in PM thickness both in clinic and experimental studies, over a prolonged period, also pointed the necessity of further study of submembrane layer.[8, 9]

Submembrane layers comprising of small cytoplasma area with a thickness up to 3-4 nm have links to PM inside. It is important to state that the above mentioned submembrane layer is not observed in all types of cells. Hyaloplasma confined in a narrow area is more adhesive and practically does not consist of any organelles. The structural elements of cytoskeleton are investigated here involve actin microphilamentous, as well as have more deeply located intermediate filaments and microtubules. Dense net of actin filaments associated with PM provide mechanical stability of cytoplasm surface layer. The association of actin filaments with febrile stabilizing proteins leads to formation of cross linking in the area of filaments crossing, which is responsible for the inflexibility of all submembrane layers. As mentioned earlier the alteration in the group of membrane compounds lipids and proteins depend on contractions generated by submembrane microphilaments. Such alterations take place by a energy requiring mechanism.[10] On the other hand actin filamentous net is able to contract because of short myosin aggregates.[11]

Proteins regulating the assembly kinetics of the cytoskeletal biopolymer F-actin are known to impact the architecture of actin cytoskeletal networks in vivo but the underlying mechanisms are not well understood.[12]

Some findings provide further evidence to support an emerging theme that cytoskeleton proteins play important roles not only for establishing membrane architecture and integrity but also for regulating the membrane protein constituents that influence the electrical properties of excitable cells.[13]

It must be noted that investigation of submembrane layer using only the method of immune fluorescence is not enough for studying structural organization of this layer as well as the structures alteration during pathological processes. The morphological studies of submembrane layer have become possible at electron microscopy resolution up to 10.000 – 20.000. Using the method of immune fluorescence microscopy, it was shown that this layer has an organized structure consisting of spherical structures, tubules and microfilaments nets.

MATERIALS AND METHODS

Reagents

Reagents used were crystalloid cardioplegic solution (Na 147 meq L^{-1}, K 19 meq L^{-1}, Ca 4 meq L^{-1}, Cl 155 meq L^{-1}, HCO_3 25 meq L^{-1}, glucose 0.2 % , pH 7.4, Mg 2 meq L^{-1}), powdered paraformaldehyde, OsO_4, Sodium cacodylate trihydrate, 96 % ethyl alcohol, acetone, Epon 812, Epon Hardener MNA, Epon Hardener DDSA, Epon accelerator DNP-30, uranyl acetate, sodium citrate, lead nitrate, photoplates. All reagent used were of analytical grade and purchased from Sigma Chemical Co. (USA).

Human Subjects

All procedures involving human subjects were approved by institutional review board/bioethical committee (Erevan State Medical University, RA) and conformed to the Legal Aspects of Research Ethics and Science in European Community directive (2001/20/EC), (IRB Approval YSMU Bioethical committee N7 by 26.04.2011).

In this study the myocardium of right atria of 10 patients with pulmonary arterial valve stenosis (PAVS) was investigated. The patients were divided into 3 groups depending on the systolic pressure of right ventricular: I group - mercury column pressure was up to 60 mm (2 patients); II group - mercury column pressure was from 60 to 100 mm (3 patients); III group - mercury column pressure was more than 120 mm (5 patients).

Biopsy material was collected during canulation process of cardiosurgical procedure of patients.

Animal studies

All procedures involving animals were approved by the Institutional Review Board\ Institutional Animal Care and Use Committee (H. Buniatian Institute of Biochemistry, Yerevan, NAS RA) and conformed to the European Communities Council directives (86\609\EC).

The material (dog myocardium) used in this study concerning PAVS was collected earlier.

For experiment involving inoculation by CCl_4, the liver of two-month-old male rates weighing 150-200 g was used. Liver toxicity was experimentally induced in Wistar male rat, weight 180-200 g, by intraventral injection of CCl_4 (3ml per each rat twice a week during 20 days).[14] The animals were maintained at the standard light and feeding conditions. A week after the last injection of CCl_4 animals were decapitated under light ether anesthesia.

Treatment of material

The bioptates taken during canulation (small pieces of the right atria) as well as at the end of experiment have immediately put in cold (4 ºC) mix of paraformaldehyde in a sodium cacodylate buffer and glutaraldehyde for 12 h with following post fixation in 1% OsO_4 solution for 2 h, dehydration in ascending series of spirits, saturation in a mixture of acetone and Epon resins of different proportions and pouring in gelatinous capsules into epon.

Obtaining of ultrathin slices and its treatment

The ultrathin slices (up to 500 Å) were made using ultracut LKB (Swedish) and Reichert (Austria). Ultrathin slices were double contrasted with uranyl acetate and sodium citrate and lead nitrate solutions.

Observation under TEM

The ultrathin slices were observed under the transmission electron microscope (Phillips CM 10) with a resolution x 20000.

Measurement of structures

The electronogramms the structures under investigation of patients and dog with valve stenosis of the pulmonary artery (VSPA), as well as the electronogramms of white rat inoculated by CCl_4 were measured by the Micro-ruler MR-1, Traceable and calculated by TED Pella ultrastructural size calculator.

Statistical analysis

Data were expresses as the mean ± S.E.M. All data were analyzed using a one-way analysis of variance (ANOVA) (SigmaStat 3.5 for Windows). Differences were considered as significant at $P < 0.05$.

RESULTS

Submembane layer of excitable cells, taken from cardiomyocytes of experimental animals, human myocardium during correction of congenital heart disease and liver hepatocytes of experimental animals, was taken as the objects for the investigation.

Examination under an electron microscope, with a resolution up to 20000, of cardiomyocytes of patients with congenital heart disease and valve stenosis of the pulmonary artery at a developed stage of disease where the process of hypertrophy takes place at the end of cytotomia process, showed the presence of three not very-large structures. These structures are in close contact to the

formed sarcolemma and to each other. Two of these structures are round-oval shaped with unchanged surface layer composed of thick filaments, each one of which in turn consists of up to three parallel thin filaments. The third structure lay between the first two and looks like leptofibrilla (Figure 1).

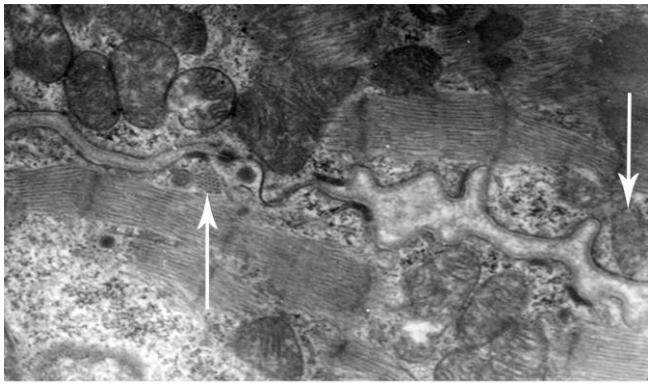

Figure 1. The ultrastructure of subplasmalemma of cardiomyocytes cytotomia of patients with VSPA**.** The presence of leptofibrilla in the centre between two round oval structures, supporting sarcolemma's arcade, is seen (x 10000).

An investigation of sarcolemma of two forming cells revealed the presence of thick short singular filaments in some parts of it, which could be residual networks.

As the destructive changes of CMC took place, the sarcolemma of scalloped windings and arcades with the destructively changed round-oval shaped structures lays one after another. These structures afforded basis for sarcolemma for its arcades and scalloped windings (Figure 2).

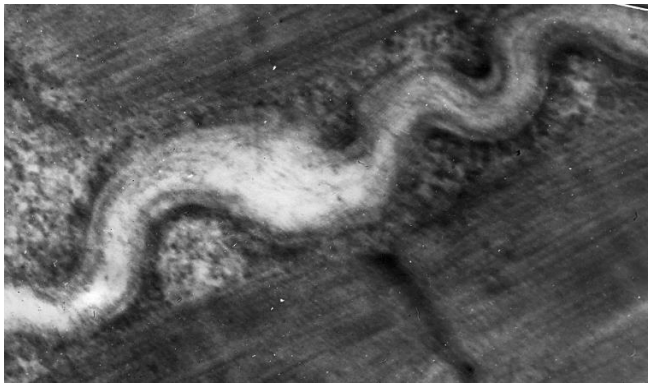

Figure 2. The ultrastructure of cardiomyocytes of patients with VSPA. The enlarged tubules with short vertical structures inside are presented. In sarcolemma's arcades are observed round-oval structures support sarcolemma (x 20000).

It must be mentioned that these structures have ability to change their sizes both in length and in width. The measurement of dimension of such structures was earlier reported.[15] It has been already shown, by TEM resolution up to 10000-20000, that these structures differ in size, depending on the injuring influence, leading to alternation in subsarcolemma layer thickness. In CMC of the above-mentioned heart disease these oval structures are up to 100-280 nm high.

At the same time small tubules are found in subsarcolemma layer of CMC closely connected to sarcolemma and composed of different short vertical structures as well as residues nets of thick filamentous. These tubules change their thickness, which in turn influence on the total thickness of subsarcolemmal layer (depending on pathological influence). The width of tubules ranges between 100-150 nm.

The experimentally induced stenosis of pulmonary arteries of dogs exhibited the presence of T-system's tubules in submembrane layer as well as noticeable alterations of thickness of T-system's tubules (Figure 3). The submembrane layer thickness varies from 50 to 100 nm.

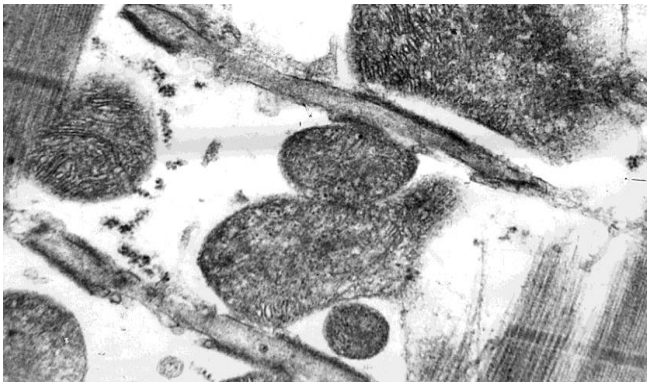

Figure 3. Ultrastructure of cardiomyocytes T-system in experimentally induced stenosis of pulmonary artery on dog model. The thickness of subplasmalemma of T-tubules is significant, as well as the presence of tubules (x 20000).

Studies of different type of cells (muscular and non muscular) have shown that as a response to pathological influence, there is a change in the thickness of submembrane layer. Such changes could be noted at electron microscope resolution up to 10000.

Inoculation by CCl_4 results in noticeable changes in hepatocyte submembrane layer thickness (Figure 4) as well as destruction of intracellular organelles resulting in the formation of shapeless aggregates consisting of mitochondria.

Figure 4. Rat liver hepatocytes inoculated by CCl_4. Significant increase in subcytolemma thickness are visible. Large round-oval structures as well as spherical structures are present (x 10000).

After repeated inoculation (twice per 20 days) of white rat liver by intraventricular injection of CCl_4, the round structures are found in submembrane layer, consisting of column of thin filaments stitched vertically by thick filaments. These structures are bound from outside by thick filaments (Figure 5). It could be suggested that a group of Mch are observed by us, however it is not like that.

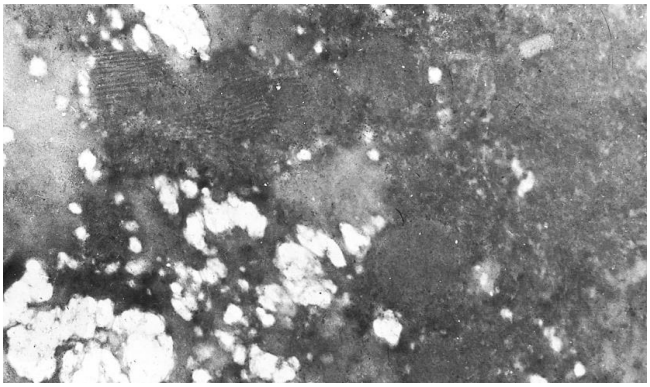

Figure 5. Rat liver hepatocytes inoculated by CCl_4 .Significantly enlargement of round oval structures are seen in large numbers (x 10000).

Prolongation of thin filaments from one side to another of the structure observed here is quite different from Mch cristae. These structure are about 480-650 nm in size. It must be noted that in hepatocytes the oval structures of the same size, as well as the shapeless masses consist of Mch, which are transformed by cristae into a type of honeycomb were found.[16]

ATP-ase activity of Mch in hepatocytes strongly decreased.[14] In the process of lyses of hepatocytes, intracellular organelles the subcytolemma layer is quite thick. The oval round structures different in size are found here. The size of these structures is up to 120-320 nm. The tubules and spherical structures consist of thick filamentous.

In a group with sodium thiosulfate injection before CCl_4 injection, the structures mentioned above up to 250-320 nm in size were also found. However the thickness of subcytolemma layer was not changed in equal measure. It could be explained by the fact that spherical structures composed of thick filaments are bigger in size compared to oval structures and are more sensitive to damaging influence.[17]

DISCUSSION

The basis of function of high specialty cardyomyocyts is the process of electromechanical linking. The coordination of all phases of contraction and relaxation of cardiomyocytes is provided by an ion transportation system. The main compounds of such system are - sarcolemma, sarcoplasmatic reticulum and mitochondria.[18] The structure of cardiomyocyte corresponds its main function. The process of electromechanical linkage takes place in it followed with further mobilization of all organelles used in this process. [19]

The data obtained from our previous studies have shown that sarcolemma's submembrane layer thickness changes primarily looks like an increasing of its electron density. Investigation of the comparison of electron high density and blurring areas of sarcolemma by the all perimeter of CMC of patients with different stages of CMC injures have shown that in a case when the prolongation of high electron density area is about 60% of sarcolemma's total length, some damaging processes are found in CMC organelles. Decreasing of this area's prolongation from 60-40% was accompanied by moderate destruction of organelles. When about 40% of total length of sarcolemma is thickened and the length of blurring areas is increased it leads to prolonged destructive changes of CMC.[20]

The cardiomyocyte has an intracellular scaffold, the cytoskeleton, which has been implicated in several cardiac pathologies including hypertrophy and failure.

The essential role of the submembrane cytoskeleton for membrane protein and cellular function is clearly illustrated by dysfunction in cytoskeleton elements in human disease.

It was earlier supposed that lateral part of cardiomyocyte salcolemma is free and that fixing of the free surface part of sarcolemma of cardiomyocyte to myofibril's on Z-line level is due to scalloped bulging at cell contraction.[19] However our studies of clinical and experimentally induced pathology have shown that submembrane layer of sarcolemma observed structures are usually not viewable at normal resolution of microscope (10000-20000), but are present in pathological samples.

These oval-round structures lay close to Z lines as well as could be observed in a space until M line. By the electron microscopic study singular structures are observed in a high quantity in sarcolemma in close contact with each other. The presence of such structures helps sarcolemma to move to scalloped bulging at cardiomyocyte contraction to form arcades at pathology.

These structures have ability to change their size which is mostly seen in reversible damages of cardiomyocytes caused by pathology.[15] As it was shown earlier defects in components of cytoskeleton affect the ability of the cell to compensate at both functional and structural levels in the long term.[21] The cytoskeleton plays the role in modulating both the electrical activity (through ion channels and exchangers) and mechanical (or contractive) activity of the adult heart. The limited visual data available suggests that the subsarcolemmal actin cytoskeleton is sparse in the adult myocyte. Cytoskeletal modulate an electro-mechanical activity in cardiac myocytes.[22] These filaments are likely to have important roles in mechanical support of ion channel function.[23]

Within epithelial cells, filamentous actin is concentrated at the plasma membrane. Its functions include structural support of the plasma membrane, establishing and maintaining cell polarity, regulation of membrane protein distribution and activity, and enhancing membrane vesicle trafficking. The actin cytoskeleton contributes the cellular pathogenesis in a number of disease states, it has been found to contribute significantly to cholangiocyte function and disease initiating structural and functional alterations in

ischemic bile ducts. The actin cytoskeleton plays a central role in the physiology and diseases of the intrahepatic bile duct.[24, 25] The important parts of choleostasis pathogenesis is decrease of hepatocyte membrane transporter, inhibition of Na^+, K^+-ATFaza and the membrane permeability, as well as destroying of hepatocyte cytoskeleton and interruption of vesicular transport.[26]

For current research of subplasmalemma layer of excitable and unexcitable cells at pathological process were chosen with different pathological conditions such as chronic pathology – narrowing of human pulmonary artery outlet and its experimental engineering on animal model, engineering of acute-chronic pathological process at rat liver using high toxical drug. As have shown the results of our study independently on pathology type both on human and animal models, submembrane layer responded by its thickness alteration and changes its structures size. The obtained data indicate that round oval structures and tubules are more stable structures compare with nets and are formed by thick filaments. At the same time it must be mentioned that under toxic influence the rate of change of size of round oval structures are more than that with the use of TiNa, which does not let the big reversal of these structures. It must be noted that at observed changes of subplasmalemma layer thickness, as well as returning of its structure to normal size need great amount of energy. However, if it takes place during the decrease of ATP-ase activity, as it was in inoculation by CCl_4, it will lead to subplazmalemma layer function disorder.[14] The process of reversibility of submembrane layer depends on energy status of cell, which can underline pathological process at tissue and organ as well.

CONCLUSION

At the bottom of the excitable and unexcitable cells plasmatic membrane thickness alteration lays changes of cell submembrane layer. Reversible and irreversible damages of its structures could be a key component in cell function disorders during pathological processes.

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