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Microtubular remodeling and decreased expression of Nav1.5 with enhanced EHD4 in cells from the infarcted heart☆

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ABSTRACT

Cardiac Na^+ channel remodeling provides a critical substrate for generation of reentrant arrhythmias in border zones of the infarcted canine heart. Recent studies show that Nav1.5 cytoskeletal- and endosomal-based membrane trafficking and function are linked to tubulin, microtubular (MT) networks, and Eps15 homology domain containing proteins like EHD4.

Aim: Our objective is to understand the relation of tubulin and EHD4 to $Na_v1.5$ channel protein remodeling observed in border zone cells (IZs) when arrhythmias are known to occur; that is, 3-h, 48-h and 5-day post coronary occlusion.

Materials methods findings: Our voltage clamp and immunostaining data show that I_{Na} density is decreased in the epicardial border zone cells of the 48 h infarcted heart (IZ_{48h}). Immunostaining studies reveal that in post MI cells the cell surface staining of $Na_v1.5$ was reduced and $Na_v1.5$ distribution changed. However, intense co-staining of $Na_v1.5$ and tubulin occurs in core planes and the perinuclear areas in post MI cells. At the same time, there were marked changes in the subcellular location of the EHD4 protein. EHD4 is co-localized with tubulin protein in discrete intracellular "highway" structures.

Significance: The distribution and expression of the three proteins are altered dynamically in post MI cells. In sum, our work illustrates the spatiotemporal complexity of remodeling mechanisms in the post-infarct myocyte. It will be important in future experiments to further explore direct links between MT, EHD proteins, and cell proteins involved in forward trafficking.

1. Introduction

After myocardial infarction, many ion channels of both the Purkinje and ventricular cells begin the process of remodeling [19]. Unlike Na^+ channels in cells from the failing heart, both the form and function of the cardiac Na^+ channel begin to remodel within hours of the occlusion of an artery [21]. In our large animal model, this may occur as early at 3 h post occlusion. Very importantly, this Na^+ channel cell remodeling provides a critical substrate for generation of reentrant arrhythmias in border zones of the infarcted canine heart [9]. Previous studies have shown the origins of arrhythmic events come from the subendocardial Purkinje and epicardial border zone myocytes. In previous work we have defined the distribution of Nav1.5 proteins and one interacting protein e.g. ankyrin G (AnkG) [12]. While we showed loss of Nav1.5 proteins, we noted no change or increase in AnkG in cells isolated from both the subendocardial Purkinje layer and the epicardial border zone. This finding was in contrast to what we had hypothesized. Ankyrin-G is not decreased but rather increased and resides under the cell membrane and intercalated discs in cells that survive in infarct border zones. While there is an obvious reserve of Na^+ channels in the heart, optimal function will depend on their location and stabilization by ankyrin-G.

If Na^+ channels are like connexin proteins where anterograde trafficking is by a targeted delivery system [5], then it is assumed that newly translated Na^+ channel proteins would be targeted directly to specific locales in the cell surface membrane. Shaw suggests that packets of channels (in his case Connexins) travel along a microtubule-based transport system [5]. After microtubules anchor at a specific protein to deliver the newly formed packets of channels (for Na channel proteins this may be AnkG [16]), "new" Na channels are inserted and function restored.

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$Na_v1.5$ protein (NacH) expression is significantly reduced in cells from the canine epicardial border zone (EBZ) 5 days post myocardial infarction (IZs) [3,12]. Functionally, sodium current density is decreased and channel function is altered in IZs. It is not known at this time whether the fundamental proteins of the forward trafficking path are preserved in cells that survive in the infarcted heart such that the Na^+ channel process of movement and relocation to cells surface/IDs can continue. Some have shown that by polymerizing tubulin, $Na_v1.5$ protein expression decreases on cell membranes, reducing $Na_v1.5$ current amplitude and modifying gating leading to cardiac arrhythmia [8]. This study implies that the polymerized tubulin might directly modify $Na_v1.5$ channel expression and function. Others have shown that correct microtubule function is critical for specific phosphorylation dependent regulation of the Na^+ channel [13].

Recently we discovered that Eps15 homology domain containing proteins serve critical roles in membrane trafficking [10,14]. Additionally, we determined that one isoform, EHD4, is differentially regulated in ventricular cells isolated from the epicardial border zone (IZ) of the post MI heart. EHD4 expression showed a significant increase in IZ cells by day 5 post-MI [14]. Moreover, this upregulation was rapid and reversible. However, the time course of change and the subcellular locale of the EHD4 protein remain unknown. The aim of this study is to understand the relation of tubulin, microtubular (MT) networks, and EHD4 to $Na_v1.5$ channel protein remodeling observed in IZs. From co-localization studies, we show that in post MI cells EHD4 is co-localized with tubulin protein in discrete intracellular structures.

2. Methods

This investigation was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animal of the National Institutes of Health (Publication No. 85-23, 1996). The protocol for all animal procedures was approved by the Institutional Animal Care and Use Committee of Columbia University (Permit Number: AAAH8905).

Healthy mongrel male dogs (24 to 26 kg, 2 to 3 years old) were used in these studies. Under isoflurane anesthesia (30 mg/kg) and sterile conditions, myocardial infarction was produced by a 2-step total occlusion of the left coronary artery using the Harris procedure [15]. The dogs were treated with lidocaine (2 mg/kg IV) if multiple ventricular beats occurred during the surgical procedure. Either 3 h, two or five days after the MI surgery, the animal was euthanized using 5–15 mg/kg IV propofol anesthesia; then a cardiectomy was performed.

2.1. Myocyte preparation

Single Ca^{2+}-tolerant ventricular cells were enzymatically dispersed from the epicardium sections with the use of a slight modification of our previously described method [1,3,17]. With this procedure, the viable cell yield was 30–40%. Two or three groups of cells were studied. One group was cells dispersed from control non-infarcted epicardium (NZs) and the others comprised the cells dispersed from the epicardial border zone (IZs) and non-infarcted zone (Remote, Rem) of the 3 h, 48 h and 5-day infarcted heart. Rem cells were always dispersed from infarcted hearts, while control NZ cells were made from same location but in naïve hearts in separate experiments on different days. In some experiments we used Rem images to compare with IZ/IZPC images, and in some experiments we used NZ/NZPC images to compare with IZ/IZPC. Both Rem and NZ/NZPC were used as Control and labelled as such. Single Purkinje cells were also enzymatically dispersed from the subendocardial Purkinje network of the normal left ventricle (NZPCs) and 3 h ($IZPC_{3h}$), 48 h ($IZPC_{48h}$) post myocardial infarction [6]. See table for list of Abbreviations used.

2.2. Protocol for staining canine ventricular myocytes and Purkinje cells

Freshly-isolated canine ventricular myocytes or Purkinje cells were seeded onto laminin-coated chamber slides. The cells were fixed for 15 min with 4% paraformaldehyde in PBS, and then permeabilized with 0.7% Triton X-100 in PBS for 20 min. The fixed and permeabilized cells were incubated with 10% normal goat serum for 30 min to block nonspecific binding. The primary antibodies were diluted in PBS containing 10% goat serum and incubated with cells overnight at 4 °C. The cells were then incubated with the secondary antibodies for 1.5 h at RT. Alexa Fluor 488- or 596- conjugated goat anti-rabbit or -mouse IgG or IgM (1:400, Molecular Probes) were used based on the primary antibodies. For negative control, the cells were treated without the primary antibodies (data not shown). The coverslip was mounted on the slide with aqueous mounting gel (Biomeda Corp, Foster City, CA).

2.3. Imaging

Confocal image stacks were captured with a Red: Nikon A1 Confocal microscope, using NIS-Elements image capture and analysis software and a TIRP super-resolution 100 × /1.45 Oil Nikon objective. Images were acquired in 1024 × 1024: laser-line1 (FITC) with an excitation wavelength λexc = 488 nm, and laser-line2 (TXRED) with an excitation λexc = 561 nm. Visualization control (LUTs) was used to avoid pixel saturation. Capture of a Z-series was used to decide top and bottom steps of a cell. In this way the surface slice (one step just below top of cell) and core slice (slice at level of nucleus) were defined. Step size (distance between slices) is 0.5 or 0.8 μm. Total slices per image varied since this depended on cell type.

2.3.1. Analysis of images

Quantifications were completed on single slice image of interest. In general one surface and one core slice were measured from each cell. The entire cell of the chosen slice was chosen as the ROI. Pearson coefficients were determined for images of interest. Colocalization analysis (Image J) was then performed by calculating % Image volume colocalized. This is the percentage of voxels which have both channel 1 (green) and channel 2 (red) intensities above threshold, expressed as a percentage of the total number of pixels in the image (including zero-zero pixels); in other words, the value of % yellow pixels in the yellow area was taken for analyses. The same threshold was set for non-infarcted and infarcted cells.

2.4. Antibodies

The antibodies used in this study include affinity-purified rabbit anti-Nav1.5 (Ig directed against peptide of DI-DII cytoplasmic loop; 1:250) [18], rabbit anti-EHD4 (1:500) and mouse anti-alpha-beta Tubulin (1:100, ThermoFisher). The immunogen for alpha-beta tubulin dimer antibody is microtubule proteins from porcine brain. Anti-rabbit IgG secondary antibody was used for the primary antibodies Nav1.5 and EHD4. Anti-mouse secondary antibody was used for the primary antibody alpha-beta tubulin.

2.5. Cell electrophysiology

Whole-cell I_{Na} was recorded using patch-clamp techniques as in [3]. Voltage-clamp experiments were performed with an Axopatch 200A clamp amplifier (Axon Instruments). For consideration of the voltage control, we used solutions containing reduced extracellular Na concentration, a maintained temperature of 19 ± 0.5 °C, and patch pipettes with resistances < 1.0 MΩ. The external recording solution (mM) contained 3 NaCl, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 127 tetraethylammonium chloride, 5 CsCl, 20 Hepes, 11 glucose, 3.0 4-aminopyridine and 2.0 $MnCl_2$ (pH 7.3 with CsOH). The internal solution contained (mM): 125 CsOH, 125 aspartic acid, 20 tetraethylammonium chloride, 10 Hepes,