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The Molecular Mechanisms of Gap Junction Remodeling

Dr. Heather S Duffy, PhD

Harvard Medical School, Boston, MA

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Localization of Connexin43 in the heart

In 1967, Revel and Karnovsky first described an aggregate of membrane associated particles which, while looking like they blocked the extracellular space, allowed the tracer Lanthanum Hydroxyde, to pass through small gaps between the aggregates, and gave this cluster of particles the name “gap junction” [1]. In the heart, they described these structures to be primarily at the long ends of the cells, identified as the intercalated disk (ID). Further studies by Page and his colleagues noted the presence of a “fuzzy coat” under the gap junctions, giving rise to the speculation that the extracellular aggregates crossed the myocyte membrane and anchored within the cell [2]. From these early studies grew a new field of research into what these aggregates were made of and how they worked. In cardiac studies, the primary component of cardiac gap junctions was identified as a 43 kD protein [3] subsequently named Connexin43 (Cx43). Studies showed that unlike the liver cells, where the hepatocyte connexin, Connexin32, was localized all over the cells[4], in the myocytes of the heart, connexins were preferential localized to the ends of cells at the intercalated disks such that images showing longitudinal sections of myocytes gave a straight line of staining that was evident at myocyte ends (Figure 1A). Transverse images show that the Cx43 does not cover the entire ends of the myocytes, rather it is positioned in a ring around the edges of the intercalated disk (Figure 1B). This pattern has been shown in mouse[5], dog [6] and human heart[7] indicating that this highly organized pattern is conserved across species. Understanding of the function of gap junctions as conduits for electrical current led to hypotheses that the localization of Cx43 in heart was important for the normal anisotropic conduction of the heart.

Interest in the role of gap junctions in heart disease was piqued when images of diseased hearts stained for Cx43 showed that cardiac pathologies were associated with a change in the normal pattern of Cx43 in the ventricular myocardium [7]. Rather than being localized at the intercalated disk, Cx43 in human hearts after a myocardial infarction was found on the sides of the myocytes, known as the lateral membranes. Being as intercalated disk localization of Cx43 was considered important for anisotropic conduction in normal heart, this “lateralization” of Cx43 was suspected to be involved in alterations of conduction in the injured heart. Subsequent studies have shown that this structural remodeling of Cx43 in the

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Correspondence to: Heather S Duffy.

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ventricular myocardium is a common result of cardiac pathology. Later studies have examined the localization of the connexins in atrial myocardium. Here the data are more confusing with some studies showing changes in connexin levels and localization following atrial fibrillation while others showed either no change or opposite results[8]. The level of structural remodeling here is not clear therefore this review will focus on the ventricular myocardium.

How Connexin43 lateralizes in the ventricular myocardium

Normal localization of Cx43 at the intercalated disk occurs through outward trafficking processes, which include interaction with the actin cytoskeleton and association with a protein complex at the ID [9]. Once at the ID, Cx43 interacts directly with the PDZ domain containing MAGUK protein Zonula Occludens-1 (ZO-1). This protein, originally thought to be uniquely associated with a different type of junction the tight junction, works to control both the size of the gap junctional plaques [10] and the localization of Cx43 at the ID [11]. So what is lateralized Cx43? There are two options. It could be new Cx43 being trafficked aberrantly in an attempt by the cell to compensate for channels at the ID that closed due to low pH or other signaling events. The other option is that it is Cx43 that had been localized at the ID but has moved away from the ID due to molecular events stimulated by cellular stress. To begin, let's look at the first option. Could this lateralized Cx43 be Cx43 trafficking outward to replace lost gap junctions and lost cellular connectivity? It is possible that in an attempt to limit loss of coupling, cells more Cx43 is trafficked to the cellular membranes for coupling. There are a few reasons that this intuitively seems less likely. First of all, under conditions of cellular stress transcription of Cx43 decreases over time as do the total protein levels in the cell. If cells were working to restore coupling it seems that there might be an increase, at least at the transcriptional level. Next, when examining cardiac myocytes stained for Cx43, internal pools of Cx43 do not increase as would be expected if more were being trafficked to cell membranes and finally, the fact that much of the Cx43 that is found in myocytes under conditions of cellular stress have phosphorylation states which are indicative of channel closure suggests that increased coupling is not occurring [12]. More importantly, studies examining the function of lateralized Cx43 have indicated a decrease in transverse coupling rather than an increase[13]. Therefore, it seems that the other option, that lateralized Cx43 is a product of loss of Cx43 from the ID, may hold more promise as an explanation. In early ischemia after only 30 minutes of coronary occlusion, Cx43 is found to be still at the ID for the most part but small amounts are now found just off to the edges of the ID. A little bit later, at 1 hr post-coronary occlusions more Cx43 is found on lateral membranes but still not completely lost from the ID. By 3 hr postcoronary occlusion, the entire lateral membrane is covered in Cx43 (Figure 1C)[11]. This step-wise increase in Cx43 on lateral membrane is suggestive of a movement of Cx43 from the ID to the lateral membranes. Thus, the hypothesis is that movement of Cx43 from the ID to the lateral membranes is a constant event following hypoxia or cellular stress that functions to remove Cx43 from the cell membranes and limit cell-cell connectivity. This has been shown to be the case in heart failure where the lateralized Cx43 was determined to be in the process of internalization at the lateral membranes of cardiac myocytes[14].

Molecular Mechanisms of Cx43 Lateralization

Studies have shown that Cx43 is tethered at the ID via interactions with the scaffolding protein ZO-1[11]. This scaffolding protein does not change its localization in ischemia or under conditions of cellular stress so how does Cx43 dissociate from ZO-1 in order to move down the lateral membranes? While all of the steps are not known, there are several events that have been identified as part of the molecular mechanism by which these proteins unhook. One of the better understood parts of the mechanism for lateralization is the role

that the non-receptor tyrosine kinase plays in this event. Under conditions of hypoxia or ischemia, activation of Src kinase occurs. Src then binds both to Cx43 to aid in channel closure but also to ZO-1 where it competes with the Cx43 binding site, thus unhooking Cx43 from ZO-1[15]. Once this interaction is lost, plaque size begins to grow but as it is no longer anchored at the ID, the entire plaque moves away from the region of high gap junction concentration at the ID to the lateral membranes, which have less Cx43. Consistent with this hypothesis, the lateralized gap junctions appear clumped in larger than normal plaques. Thus, when we take images of fluorescently stained Cx43, we see a snapshot in time of the Cx43 that was at the ID but is not moving down the lateral membrane. Once there, plaques begin to be internalized via clathrin mediated events for subsequent degradation[16].

Another potential mechanism for lateralization recently came out suggesting that acetylation of Cx43 may play a role[17]. Colussi et al were interested in the effect of increase acetylase P300/CBP-associated factor (PCAF). In a model of Duchenne cardiomyopathy (mdx), they found that Cx43 was lateralized and co-immunoprecipitated with PCAF even in the absence of an activation of c-Src. These authors went on to show that N^ε-lysine acetylation of Cx43 occurred in the mdx hearts and was predominantly found on the lateral membranes where it associated with PCAF. Additionally, heart samples from patients with Duchenne's muscular dystrophy were found to have lateralized Cx43. These interesting data do not give mechanisms of lateralization by N^ε-lysine acetylation but show clearly that activation of c-Src and its interactions with Cx43 and ZO-1 are not the only mechanisms by which Cx43 becomes lateralized.

Functional Implications of Cx43 Lateralization

Conduction through the ventricular myocardium begins with His bundle activation of the Purkinje System on the endocardial surface of the heart. Current flow from the Purkinje fibers activates ventricular myocytes which then transfer current, anisotropically down the longitudinal aspect of the cells. This anisotropic conduction driven by the His-Purkinje system is important for normal cardiac function causing contraction to begin at the *apex* of the heart and move upwards to the *base* allowing for efficient ejection of blood from the ventricles. This conduction occurs due to a number of factors including cell shape as well as the placement of Cx43 at the longitudinal ends of the cardiac myocytes where it forms low resistance conduits to preferentially transfer current passively in the longitudinal direction. So, what then happens to conduction when Cx43 becomes lateralized? Based on studies identifying Cx43 along lateral membranes by immunohistochemistry, which gives no information about function, the initial hypothesis was that these junctions could passively transfer current in the transverse direction, across lateral membranes, decreasing the anisotropic ratio and slowing conduction. To do this, the lateral gap junctions would need to be open and able to pass current. Initial studies on functionality were done on isolated cells, using patch clamp studies of cells from the Epicardial border zone in a canine model of permanent coronary occlusion. These studies showed that the cells from this region were less likely to engage in transverse conduction[13], rather than more likely. Additionally, there is an overall loss of Cx43 protein in these areas, adding to the decrease in gap junction function[18]. In both the cultured cells and the intact heart the loss of coupling was found to occur primarily in the transverse direction, although some loss of coupling in the longitudinal direction was also noted.

In addition to the structural remodeling of connexins in the diseased heart, functional remodeling of connexins also occurs by regulation of the phosphorylation status of the channels. The major cardiac connexins, Cx40, Cx43 and Cx45, have been shown to be regulated by phosphorylation primarily of serine/threonine residues although Cx43 in

particular has been shown to be phosphorylated on tyrosine residues as well[19]. It was previously believed that loss of phosphorylation at some sites on the carboxyl terminal domains of connexins, such as S252 on Cx43, causes channel closure and that a downward shift in mobility of the Western blot bands could be used as a reliable indicator of channel closure but newer evidence indicates other sites such as S368 remain open when dephosphorylated and phosphorylation leads to the closed confirmation[19]. These data suggest that relying on the mobility shift in Western blot bands is not optimal for determining the open or closed status of this particular channel. There are many more examples of functional remodeling of connexins by phosphorylation that are beyond the scope of this review but the reader is directed to reviews on the topic for more in depth information[19–21].

In conclusion, Cx43 lateralization occurs in many cardiac pathologies although the mechanisms for these changes are not known. To date, there have not been comprehensive studies on the molecular mechanisms by which Cx43 lateralizes except in the case of ischemic injury. Further studies on Cx43 localization and lateralization in heart failure are needed to determine if the molecular mechanisms of lateralization are uniform across injuries or if differing pathologies cause different molecular events to occur which translate into the same phenotype, lateralized Cx43. Another important corollary would be to understand the physiological implications to either inhibiting Cx43 lateralization or reversing the process following injury. Being as gap junctions are important for normal electrical propagation in the heart, there has been great interest in finding therapies that either limit or reverse Cx43 lateralization in the injured heart. These studies have had mixed results. While the goal has been to maintain coupling, which has been achieved, this has come at the cost of an increase in infarct size. It is unclear exactly what molecules are passing through gap junctions to illicit this response but the end result of a larger infarct is not a desirable outcome. One caveat is that most of the studies that have examined the maintenance of Cx43 at the ID in injury have done so with models that maintain connexins at the ID during the entire ischemic insult. Perhaps this trick is in the timing. For example, if Cx43 gap junctions were maintained in the open state during reperfusion, to limit reperfusion arrhythmias, then allowed to close to limit infarct size perhaps a balance between arrhythmias and infarct size could be struck. Another option would perhaps be to allow Cx43 gap junctions to close early in ischemia but over time begin therapies to keep junctions open to reduce further arrhythmias. While there are no data on either condition, I'd place my bets on the former treatment being more efficacious due to the presence of activated fibroblasts later in the course of disease. These fibroblasts have been shown to form fibrotic expanses that intercalate between myocyte bundles and physically limit connectivity between myocytes by limiting sites of cell-cell contact[22]. Thus, keeping the junctions open at later stages would not help being as the myocytes have limited contact with each other at this time. Additionally, we need a deeper understanding of the molecular events that underlie Cx43 lateralization in heart failure and the effect of maintenance of gap junctions under these conditions. Being as heart failure leads to arrhythmias, maintenance of coupling may provide relief and without the dying infarct region to be concerned about, this treatment may have fewer negative consequences.

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Figure 1.

Immunolocalization of Cx43 in atrial myocytes. Cx43 (red) is localized to the intercalated disk of cardiac myocytes with ZO-1 (green) which, in longitudinal view (A) gives the well described image of lines of Cx43 staining at the ends of the cells with yellow areas of overlap between Cx43 and ZO-1. When the cells are viewed from the transverse direction, on the other hand, it becomes clear that the majority of Cx43 is found not covering the entire surface of the intercalated disk but preferentially in a ring formation around the edges of the disk while ZO-1 covers the entire disk (B). Ischemia causes rapid change in Cx43 localization such that a majority of protein is now found on the lateral membranes of the cardiac myocytes and co-localization with ZO-1 at the intercalated disk is limited.