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The Noncanonical Functions of Cx43 in the Heart

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Abstract There is abundant evidence showing that connexins form gap junctions. Yet this does not exclude the possibility that connexins can exert other functions, separate from that of gap junction (or even a permeable hemichannel) formation. Here, we focus on these noncanonical functions of connexin43 (Cx43), particularly in the heart. We describe two specific examples: the importance of Cx43 on intercellular adhesion, and the role of Cx43 in the function of the sodium channel. We propose that these two functions of Cx43 have important repercussions on the propagation of electrical activity in the heart, irrespective of the presence of permeable gap junction channels. Overall, the gap junction–independent functions of Cx43 in cardiac electrophysiology emerge as an exciting area of future research.

Keywords Arrhythmia · Cell–cell adhesion · Connexin43 · Heart

Introduction

Sixty years ago, Silvio Weidmann published his classic article on "The Electrical Constants of Purkinje Fibers." In it, he beautifully demonstrated that electrotonic propagation in cardiac tissue extends well beyond the size of a single cell (Weidmann 1952). His observations provided the physiological evidence that cardiac cells are electrically coupled via low-resistive pathways. Electron microscopic observations followed, culminating with the elegant work of Revel and Karnovsky (1967) showing that at the site of close appositional membranes in the cardiac intercalated disc, the membranes were not fused. Instead, the membranes were separated by a gap, traversed by junctions. These findings led Revel to later coin the term *gap junctions.* The demonstration that gap junctions are formed by oligomerization of connexin proteins established gap junction formation as the key function of connexin molecules. Yet the fundamental importance of connexins in intercellular communication does not exclude the possibility that connexins may exert other functions, separate and independent from that of gap junction formation. This is hardly a novel concept. Twenty years ago, Ross Johnson and his colleagues reported a very important discovery: Fab fragments of antibodies to the extracellular domain of connexin43 (Cx43), the most abundant connexin in the heart, inhibits adherens junction assembly in cells in culture (Meyer et al. 1992). This unexpected finding has been followed by others, showing that Cx43 is not only a pore-forming protein that allows ions and small molecules to move between cells (see, e.g., Danik et al. 2008; Jansen et al. 2012a, b; Francis et al. 2011; Soder et al. 2009). Our understanding of the molecular nature of connexins and their function has expanded enormously since Ross Johnson, Weidmann, Revel, Bennett, Gilula, Goodenough, and many other giants of science first paved the way. Yet a number of interesting questions about the role of connexins in biology remain unanswered, while other concepts that seemed established are now challenged by novel experimental results

Here, we will dwell on what we call the noncanonical functions of Cx43—that is, functions that go beyond that of

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gap junction formation. Although our discussion will center on Cx43 and its role in heart function, these issues likely extend to the functions of other connexins and in other organs. Of the many interesting angles that apply to this topic, we will cover only two aspects: intercellular adhesion and sodium channel function. These functions are described from the point of view of Cx43 as a component of the protein interacting network (the interactome) that populates the cardiac intercalated disc.

The Intercalated Disc as the Site of a Protein Interacting Network

Cardiac myocytes are highly differentiated, specialized, and compartmentalized cells. Proteins organize in defined microdomains. Slight changes in the position of a protein within its domain can bring about a major disruption in function (see, e.g., Nikolaev et al. 2010). Connexins occupy a subcellular region called the intercalated disc. This electron dense structure is located at the point where two cardiac cells meet end to end. In its classical definition, the intercalated disc is composed of three electron dense structures: gap junctions, desmosomes, and adherens junctions. The latter two are involved in mechanical coupling between cells. Desmosomes couple to intermediate filaments (desmin, in the case of the heart), whereas adherens junctions anchor N-cadherins to the actin cytoskeleton. A mixed desmosome/adherens junction structure, dubbed the *area composita*, is also present in the adult mammalian heart (Franke et al. 2006). Originally these structures were considered separate and independent from each other. Recent data suggest this not to be the case. Furthermore, the advent of immunofluorescence techniques brought about the demonstration that other molecules, not classically considered junctional, are also present at the cell end and in fact colocalize with junctional molecules. Of particular interest are two ion channel proteins fundamental to normal cardiac electrophysiology: the sodium channel alpha subunit, Nav1.5, and the potassium channel protein $K_V1.5$. For a number of years, each of these channels, and their accessory proteins, were studied as independent entities. Yet recent data show that there is extensive cross-talk at the intercalated disc, and that this cross-talk extends to interactions between complexes previously seen as being independent (Fig. 1). As such, loss of expression of plakophilin-2 (PKP2), a desmosomal molecule, affects gap junction–mediated coupling (Oxford et al. 2007) as well as sodium channel function (Sato et al. 2009); loss of N-cadherin expression affects gap junctions (Li et al. 2005) and also the function of $K_V1.5$ channels (Cheng et al. 2011); loss of intercellular contact leads to a decrease in sodium current (Lin et al. 2011); expression of ankyrin-G (AnkG), a protein

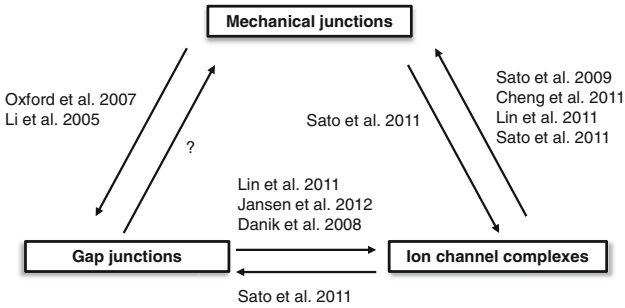

Fig. 1 Diagram indicating cross-talk between gap junctions, mechanical junctions, and ion channel complexes. Gap junctions refers to Cx43, while mechanical junctions includes desmosomes and adherens junctions (N-cadherin). Ion channel complexes refers primarily to sodium channel complex and $K_V1.5$. Citations correspond to experimental evidence in cardiac preparations that support the interaction described

associated with the sodium channel complex (Lowe et al. 2008), is necessary for proper intercellular adhesion strength and for proper electrical coupling (Sato et al. 2011); finally, expression of Cx43, a protein previously associated only with gap junctions, is in fact required for the normal function of sodium (Jansen et al. 2011) and potassium currents (Danik et al. 2008). When taken together, the evidence suggests that the intercalated disc is not a site where independent molecules reside, but rather the host of an interactome—a protein-interacting web that involves molecules relevant to excitability, propagation, and mechanical coupling between cells.

Cx43 and Intercellular Adhesion

The possible interaction between connexins and mechanical junctions in the heart was highlighted by the findings of Jeff Saffitz and his colleagues. These investigators examined the hearts of patients afflicted with arrhythmogenic right ventricular cardiomyopathy, a disease related to mutations in proteins of the desmosome, and demonstrated a consistent loss of gap junction plaques from the intercalated disc (Kaplan et al. 2004). Follow-up work showed that loss of expression of the desmosomal protein, PKP2, leads to a loss of Cx43 from the site of intercellular contact and an $\sim$50 % decrease in the extent of dye transfer between cells (Oxford et al. 2007). These and other studies strongly supported the notion that mechanical junctions affect gap junctions. On the basis of the early work of Meyer et al. (1992), we have now asked a reciprocal question: is Cx43 expression necessary to maintain intercellular adhesion strength? To address this question, we implemented a dispase assay, whereby the contact between the cells and the extracellular matrix is disrupted by the use of dispase. If adhesion between cells is strong, the layer

lifts as one sheet (cells remain attached to one another). If cell–cell adhesion is weaker, the sheet fragments. Thus, the more fragments, the weaker the mechanical coupling. Using this method, we recently showed that loss of Ank-G expression (a protein that scaffolds for the sodium channel complex) causes a decrease in mechanical coupling between cells (Sato et al. 2011). Here, we applied this method to compare two cell populations: HEK293 cells that endogenously express Cx43; and a stable HEK293 line where we used lentivirus to permanently silence Cx43 expression. Three groups are compared: untreated (UNT), treated with a virus that contains a nonsilencing construct (ϕshRNA-Cx43), and a third group where Cx43 expression was prevented (shRNA-Cx43). Western blot analysis revealed the loss of Cx43 expression in the corresponding group (Fig. 2), and loss of Cx43 expression brought about a loss of intercellular adhesion strength, represented by a significant increase in the number of fragments detected 90 min after dispase addition. Thus Cx43 expression is relevant to mechanical coupling. Whether this effect is consequent to gap junctions being a physical element that provides intercellular adhesion, or whether the result involves intermolecular interactions between Cx43 and components of the mechanical junctions, remains to be determined. The results do show that intercellular adhesion strength is a function of Cx43 that extends beyond the formation of a low-resistive pathway between cells.

Cx43 Is Necessary for Sodium Channel Function

Figure 1 also shows an arrow connecting Cx43 to the sodium channel complex. This is an exciting and novel finding regarding the noncanonical functions of Cx43 in the heart. Indeed, in the classical description, sodium channels provide the current that is necessary for the generation of an action potential in most cardiac cells, whereas Cx43 forms gap junctions, the channels that allow for that electrical charge to move between one cell and the next. This description then separates the type of channel, with its function: sodium channels are responsible for cell excitability; gap junctions allow cell–cell passage of charge. In a recent study, however, we reported that loss of Cx43 expression brings about a loss of sodium current amplitude (Jansen et al. 2012a). In other words, the molecule necessary for making the gap junctions is actually necessary to maintain the complex in charge of generating the action potential. This means that loss of Cx43 expression is in fact a double-edge sword: not only will the path between cells be disrupted, but also the amount of charge that is generating by the excited cell will decrease. Loss of Cx43 expression, as it happens in a number of pathological cardiac conditions (Desplantez et al. 2007; Akar et al. 2007; Chkourko et al. 2012; Kalcheva et al. 2007; Qu et al. 2009), can have complex deleterious effects on propagation.

Novel Roles of Cx43 in Cardiac Propagation: Sodium Current and the Intercellular Space

These findings come at a time when the classical description of cardiac propagation deserves to be reviewed. Early models based on the principles of continuous cable theory represented gap junctions as passive resistors providing the only means for passage of charge between cells. When incorporated into a model of cardiac propagation, these equations predicted that reductions in gap junction–mediated communication would bring about a concomitant reduction in conduction velocity. The first serious challenge to this notion came from experiments demonstrating that 50–80 % loss of Cx43 expression in the heart causes a decrease in junctional conductance between cells (Yao et al. 2003), but not a decrease in conduction velocity (Morley et al. 2000; Danik et al. 2004). Are gap junctions, then, the only path for transfer of charge between cardiac cells? What is the missing element (missing from the equations) that preserves propagation when gap junction–mediated conductance decreases?

Although a conclusive answer has not yet been found, it seems pertinent to remain open to models that, though less conventional, may better represent what happens at the site of contact between cells. We refer in particular to the proposed electrical field mechanism of cardiac propagation. This model postulates that the large inward sodium current in the proximal side of an intercellular cleft can generate a large negative extracellular potential within the cleft, effectively depolarizing the membrane of the distal cell, activating its sodium channels and allowing for propagation to continue downstream, even in the absence of functional gap junctions (Sperelakis 2002; Hand and Peskin 2010; Mori et al. 2008). Although this mechanism would play an insignificant role when gap junctions are present and functional, it would become crucial to maintain propagation when gap junctions close or when connexins are lost.

The effect of electric fields, also known as ephaptic interactions, has been extensively investigated in the nervous system. These nonsynaptic mechanisms play a significant role in neuronal function and can mediate neuronal synchrony on a fast scale compared to ionic and chemical mechanisms that operate on a much slower scale (Jefferys 1995). In this context, recent studies of the presence of electrical synapses in the mammalian central nervous system have described the chemical transmission through Cx36 in neurons of the mesencephalic trigeminal nucleus. Although in these cells the fraction of opened channels is small, the sodium and potassium conductances enhances

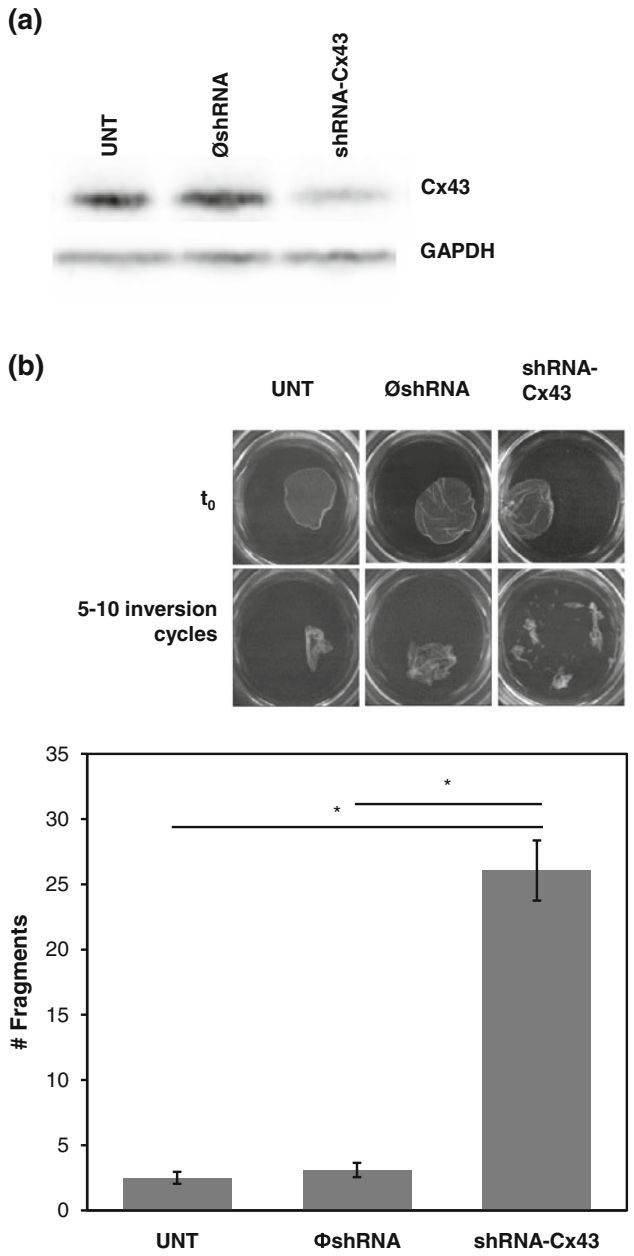

Fig. 2 Loss of Cx43 induces loss of intercellular adhesion strength. **a** Western blot for Cx43 in HEK293 cells untreated (UNT), treated with a virus that contains a nonsilencing construct (ϕshRNA-Cx43) and silenced for Cx43 (shRNA-Cx43). GAPDH was used as loading control. **b** Dispase assay. Cells were treated with 2.4 U/mL dispase for 90 min to disrupt attachment to extracellular matrix. Images were taken before (t_0) and after 5–10 inversion cycles. *Bars* show number of fragments after subjecting monolayers to 5–10 inversion cycles. $n = 10$–20, $p < 0.001$

the electrical coupling leading to a strong synchronization of these neurons (Curti et al. 2012).

Under the concept of the electric field mechanism, propagation is dependent on the subcellular distribution of sodium channels because their density, specifically at the intercalated disc, is critical to the generation of the electrical field in the intercellular cleft (Tsumoto et al. 2011). In this context, it is important to mention our recent results (Lin et al. 2011) showing that (a) sodium current amplitude is larger in the area of the intercalated disc; (b) steady-state inactivation of sodium channels located in the middle of the cell is shifted toward more negative values; as such, the burden of excitation during propagation falls on the channels at the intercalated disc; and (c) sodium current density is larger in cells that remain paired. These results are complementary to those of Petitprez et al. (2011), showing that there are two separate pools of Nav1.5 in the heart: one at the intercalated disc and a separate one associated with the costameres. Our data demonstrated that, together with the segregation of channels by subcellular regions, there is also a segregation of function; here, we further propose that the function of Nav1.5 at the intercalated disc is determined, at least in part, by Cx43.

The electric field mechanism also requires a preservation of the dimensions of the intercellular space. Experimental values for this variable are less solid. Currently, we are using high-pressure freezing methods and tomographic electron microscopy to obtain images of the intercellular space with a high level of structural preservation (Delmar and Liang 2012). Using these methods, we have began to collect images that allow us to make accurate measurements of the intercellular space under conditions where Cx43 is reduced, either by genetic manipulation (e.g., in Cx43-deficient hearts) or by disease. In this context, it seems pertinent to remind the reader of the discovery of Ross Johnson and his colleagues 20 years ago: connexins are important for intercellular adhesion (Fig. 2). It would follow that diseases that cause loss or remodeling of Cx43 would lead to an increased separation of the cells at the intercalated disc. From the point of view of electrophysiology, this would become an important challenge to the preservation of propagation between cells.

Conclusions

We have discussed two noncanonical functions of Cx43: preservation of cell–cell adhesion and preservation of sodium current amplitude in cardiac cells. We have also argued in favor of a model of cardiac propagation where cell–cell transfer of charge occurs not only by the flow of current through gap junction channels but also, by an electric field mechanism that relies on (a) a tight intercellular gap and (b) the accumulation of functional sodium channels at the intercalated disc. Thus, we speculate that under conditions of poor gap junction–mediated electrical coupling, propagation can be maintained via the electrical field mechanism, but only if sodium current properties, and a narrow cleft, are preserved. As such, we propose that the

Cx43-dependent loss of sodium current and perhaps a Cx43-dependent increase in the size of the intercellular gap are critical to propagation failure resulting from reduced Cx43 abundance. Cx43 may play a key role in intercellular communication not only directly, by forming gap junctions, but also indirectly, by maintaining a high sodium current density at the intercalated disc, and a narrow intercellular cleft for the transfer of activation. The gap junction–independent functions of Cx43 in cardiac electrophysiology emerge as an exciting area of future research.

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