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Relating specific connexin co-expression ratio to connexon composition and gap junction function

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ABSTRACT

Cardiac connexin 43 (Cx43), Cx40 and Cx45 are co-expressed at distinct ratios in myocytes. This pattern is considered a key factor in regulating the gap junction channels composition, properties and function and remains poorly understood.

This work aims to correlate gap junction function with the connexin composition of the channels at accurate ratios Cx43:Cx40 and Cx43:Cx45.

Rat liver epithelial cells that endogenously express Cx43 were stably transfected to induce expression of accurate levels of Cx40 or Cx45 that may be present in various areas of the heart (e.g. atria and ventricular conduction system). Induction of Cx40 does not increase the amounts of junctional connexins (Cx43 and Cx40), whereas induction of Cx45 increases the amounts of junctional connexins (Cx43 and Cx45). Interestingly, the non-junctional fraction of Cx43 remains unaffected upon induction of Cx40 and Cx45. Co-immunoprecipitation studies show low level of Cx40/Cx43 heteromerisation and undetectable Cx45/Cx43 heteromerisation. Functional characterisation shows that induction of Cx40 and Cx45 decreases Lucifer Yellow transfer. Electrical coupling is decreased by Cx45 induction, whereas it is decreased at low induction of Cx40 and increased at high induction.

These data indicate a fine regulation of the gap junction channel make-up in function of the type and the ratio of co-expressed Cxs that specifically regulates chemical and electrical coupling. This reflects specific gap junction function in regulating impulse propagation in the healthy heart, and a pro-arrhythmic potential of connexin remodelling in the diseased heart.

1. Introduction

Gap junction channels (GJCs) directly link neighbouring cells enabling intercellular chemical and electrical coupling. A GJC consists of two docked hexameric connexons [1,2] composed of connexins (Cxs). In human, 21 Cxs genes have been identified and most tissues co-express more than one Cx type [3]. Consequently different GJCs would be possible [4]: connexons composed of one Cx isotype are referred to homomeric, and heteromeric when composed of different Cxs. When identical connexons pair, GJCs are termed homotypic while GJCs formed by different connexons are called heterotypic. If two Cxs can freely assemble, 12 heteromeric and 2 homomeric connexons, and potentially 196 types of GJCs could be formed [4,5]. This would predict a probability of 2/196 homomeric homotypic GJCs in a cell co-expressing two types of Cxs [4,5].

Cardiomyocytes co-express connexin 43 (Cx43), Cx40 and Cx45 in developmental and tissue specific patterns [6], which is hypothesised to determine the conduction patterns that govern the normal heart rhythm [7]. In the diseased human heart and animal models of heart disease, alterations of Cxs localisation and expression (termed remodelling) are recognized as a major pro-arrhythmic contributor [6]. For example, in the failing left ventricle Cx43 is heterogeneously reduced [6,8] whereas Cx45 is up-regulated [9], and in ischemic cardiomyopathy endocardial Cx40 is up-regulated [8]. Similarly in atrial fibrillation alterations of Cx43 and Cx40 co-expression and distribution have been reported, however with inconsistent results (reviewed in [6,10,11]). These changes are postulated to alter action potential propagation.

To examine the compatibility of connexons made of Cx43, Cx45 and Cx40, transfected cell models have been studied. Whereas evidence of heterotypic Cx43/Cx45 GJCs was found [4,12–16], ambiguous results have been obtained for Cx43/Cx40 GJCs [7,13,14,16–19]. Functional studies by double patch clamp suggest that the majority of GJCs are of

Abbreviations: RLE, rat liver epithelial; ind40, stable transfected cells for Cx40; ind45, stable transfected cells for Cx45; indGFP, stable transfected cells for green fluorescent protein; PonA, ponasterone A; LY, Lucifer Yellow; Cxs, connexins; Cx43, connexin 43; Cx45, connexin 45; Cx40, connexin 40; GJCs, gap junction channels.

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mixed composition and that homomeric–homotypic GJCs are rare [4,12,13,15,20], which is in agreement with the theoretical considerations [4,5]. A limit of these cell models is the lack of control over the ratios of the co-expressed Cxs which likely affect the GJCs make-up. Furthermore double patch clamp on cell pairs represents a setting that does not exist in vivo or in cultured monolayers.

To gain more insight into the structural and functional consequences of Cx co-expression ratios, we engineered a rat liver epithelial cell line (RLE) with endogenous Cx43 expression that upon addition of ponasterone A expresses inducible levels of Cx45 or Cx40 [21,22]. With this system different accurate co-expression levels and ratios of Cx45:Cx43 and Cx40:Cx43 have been quantified and functional studies (dye transfer, electrical coupling) performed. At similar expression level of co-expressed Cx43/Cx40 and Cx43/Cx45, distinct junctional and non-junctional levels were found, and heteromeric connexons are formed in low (Cx43/Cx40) and undetectable amounts (Cx45/Cx43). Distinct electrical cell–cell coupling was observed: Cx43/Cx45 co-expression leads to a lower electrical coupling than non-induced cells, similar at each Cx45:Cx43 ratio, whereas the electrical coupling in Cx43/Cx40 co-expression depends on the level of induction of Cx40. Altogether our data suggest a distinct GJCs make-up in function of the type and the ratio of co-expressed Cxs.

2. Materials and methods

As suggested in the instructions for authors, detailed materials and methods are described in the online supplement.

2.1. Culture and transfection

Rat Liver Epithelial cells (RLE) were stably transfected to express the ecdysone transcription factors and to express a bi-cistronic mRNA coding for the native mouse Cx40 or Cx45 and the hygromycin resistance. Detailed protocols for transfection and culture of RLE and the criteria used to select the clones (ind40, ind45 and indGFP) can be found in the online supplement. HL1-6 culture conditions are the same as described in [23,24].

2.2. siRNA transfection

Transfection of siRNA to Cx43 (2 μM) was done by electroporation. The sequence used and electroporation parameters are detailed in the online supplement.

2.3. Sample preparation, Western blotting and relative comparison of connexin expression

Sample preparation, separation of the junctional from the non-junctional fraction using Triton X-100, and immunoprecipitation are described in detail in the online supplement.

To compare the relative amounts of Cx40, Cx43 and Cx45 we used lysates of HeLa cells transfected with the native Cxs and with our V5/6-His tagged Cxs [21,25,26]. First, the tagged Cxs lysates were diluted to give equivalent reactivity with the anti-V5 antibody. Then the native Cx lysates were diluted to give equivalent reactivity to the tagged Cxs lysates with the specific anti-Cxs antibodies directed against internal sequences of each Cx, anti-Cx40 S15C (R83) [27], anti-Cx43 MAB3068, Chemicon and anti-Cx45 Q14E (mab19-11-5) [28]. These native Cxs lysates containing the same relative amounts of Cx40, Cx43 and Cx45 were then loaded into the gels used for relative quantification thereby allowing us to compare Cxs to each other, 100% representing the relative expression of Cx43 in ind40, ind45 and indGFP transfectants without induction. Although, the anti-Cx40 S15C (R83) against the internal site was about 5 to 10 times less sensitive than the anti-Cx40 (C20, Santa Cruz) that we used in our experiments, it was useful to calibrate the Cx40 lysate since, as expected, binding of the anti-Cx40 (C20, Santa Cruz) directed against the C-terminus of Cx40 is affected by the presence of the C-terminal V5/6-His tag.

2.4. Immunofluorescence labelling

Single and double labelling protocols and the antibodies used for these experiments are detailed in the online supplement.

2.5. Lucifer Yellow dye transfer

Dye transfer experiments with Lucifer Yellow were done by scrape loading. Detailed procedure and quantification methods are described in the online supplement.

2.6. Double patch clamp experiments

Electrical recordings were performed by applying the dual voltage clamp method on cell pairs as described in [4]. Briefly a gradient of membrane potential Vj = 10 mV was applied between adjacent cells which permits to record the junctional current Ij that diffuses through the GJCs. The amplitude of Ij was measured and the junctional conductance $G_{j,0}$ was calculated in each experimental condition (non-induced and induced Cx expressions). Details of the procedures, solution used and analysis are described in the online supplement.

2.7. Statistical analysis

Comparison of the quantities of total, junctional and non-junctional fractions for each Cx was done by one way ANOVA.

3. Results

3.1. Specific Cxs distribution

RLE cells endogenously expressing Cx43 were maximally induced to express Cx40 or C45. Immunolabelling experiments show that Cx43 was homogenously distributed along the cell–cell interfaces (Fig. 1A) as in the non-induced cells (not shown). Upon induction of Cx45 (ind45 cells; Fig. 1A, top panels) and Cx40 (ind40 cells; Fig. 1A, bottom panels) we observed a junctional co-localisation of the two Cxs, and a non-junctional Cx40 and Cx43 co-localisation consistent with the Golgi apparatus where Cx43 oligomerises before transfer to the plasma membrane [29] while the cytoplasmic Cx45 appears diffuse around the nuclei. This diffuse distribution is also observed in the co-culture ind45/indGFP experiment (Fig. 1B, top left panel) and has never been observed without Cx45 induction (data not shown) or in the other transfectants (e.g. indGFP or ind40 cells — Fig. 1B) indicating a specific labelling of Cx45 accumulating in an undefined cytoplasmic compartment before transfer to the plasma membrane.

The compatibility between cells expressing only Cx43 (i.e. indGFP cells) and cells co-expressing Cx43 + Cx45 or Cx43 + Cx40 was examined by co-culturing both types of cells (Fig. 1B). As seen in Fig. 1B, Cx45 was present at the junctional membrane between the ind45 and indGFP (Fig. 1B; top left panels) suggesting that, as already reported, Cx43 and Cx45 form heterotypic GJCs [4,7]. However, Cx40 remains undetectable between ind40 cells and indGFP cell (Fig. 1B; left bottom panels). This finding, in agreement with the studies of Elfgang et al. [14,18], indicates that Cx40 even co-expressed with Cx43 docks poorly if at all with homomeric Cx43 GJCs. Conversely ind45 and ind40 display abundant Cx43 labelling between the two cell types (Fig. 1B right panels). These data suggest that homomeric and/or heteromeric connexons made of Cx45 can dock with homomeric Cx43 connexons, but that homomeric or heteromeric connexons composed of Cx40 have a low affinity for homomeric Cx43 connexons and are undetectable by immunofluorescence [30]. Therefore, the majority of GJCs between ind40 and indGFP cells are likely homomeric–homotypic Cx43.