LOCALIZATION OF ANKYRIN G, NA_V AND KCNQ1 CHANNELS TO NEURO-CARDIAC JUNCTIONS.

Olga G. Shcherbakova.

Petersburg Nuclear Physics Institute named after B.P. Konstantinov, National Research Center "Kurchatov Institute", Department of Molecular and Radiation Biophysics. Orlova Roscha, Gatchina, Leningrad district, 188300, Russia.

Abstract

Cardiac performance is regulated by input of sympathetic nervous system. Junctions between sympathetic ganglion neurons (SGN) and cardiac myocytes are unique cell-cell contacts designed for the efficient regulation of heart rate and contractility by the autonomic nervous system. In the present study, we report that scaffold protein ankyrin G is localized to the postsynaptic sites in co-culture of cardiac myocytes and SGN. Consistent with roles of ankyrin G in targeting Na_v channels to excitable domains in neurons and cardiac myocytes, we observed increased density of Na_v channels at the neuro-cardiac junctions in co-cultures of cardiac myocytes and SGN. We also have found that KCNQ1 channels are present in increased density at the specialized zones of contacts of cardiac myocytes and SGN. An increased density of Na_v and KCNQ1 channels at the neuro-cardiac sympathetic junctions may have an important role in modulation of cardiac action potential by neuronal input.

Introduction

The sympathetic nervous system plays a central role in the cardiovascular response to acute stress by increasing heart rate and contractility (Armour and Ardell, 1994). Little is known about molecular organization of functional contacts of cardiac myocytes and sympathetic neurons. Earlier, we provided the first evidence for molecular specialization in the postsynaptic membranes of innervated cardiac myocytes in co-culture with sympathetic ganglion neurons (SGN) (Shcherbakova et al., 2007). We have demonstrated that the myocyte membrane develops into specialized zones that surround contacting axons and contain accumulations of the scaffold proteins SAP97 and AKAP79/150 but are deficient in caveolin-3. The β 1 ARs are enriched within these zones, whereas β 2 ARs are excluded from them after stimulation of neuronal activity. The results indicate that specialized signaling domains are organized in cardiac myocytes at sites of contact with sympathetic neurons. Direct coupling between neurotransmitter releasing sites and

effectors cardiomyocytes membranes was further confirmed by other authors (Prando et al., 2018).

It is well established, that the input of sympathetic nervous system mediated by β1-adrenergic receptor activation, results in increase in cardiac contractility and shortening of cardiac action potential duration (APD). Cardiac action potential is formed by the finely balanced activity of multiple ion channels and transporters. Localization and targeting of the cardiac ion channels are critically important for efficient action potential initialization and propagation through myocardium. Ankyrin polypeptides play critical roles in ion channel and transporter targeting in excitable cells. In cardiac myocytes ankyrin-G associates with Nav1.5, primary ion channel responsible for the upstroke of cardiac action potential. It has been demonstrated that the mutation in $Na_v1.5$ channel that abolishes binding of $Na_v1.5$ to ankyrin-G and prevents accumulation of $Na_v1.5$ at cell surface sites in ventricular cardiomyocytes, cause Brugada syndrome, which is a severe form of arrhythmia leading to sudden death (Mohler et al., 2004). In the present brief research communication, we report that scaffold protein ankyrin G localizes to the neuro-cardiac junctions in co-culture of cardiac myocytes and SGN. We also observed that Na_v channels are present in increased density at the specialized zones of contacts of cardiac myocytes and SGN.

The input of sympathetic nervous system, mediated by β - adrenergic receptor activation, increases the slow outward potassium ion current (I_{KS}) to accelerate repolarization and shorten the ADP (Kass and Wang, 2000; Marx, 2002). In human ventricular myocytes, KCNQ1-KCNE1 channels generate I_{Ks}, a slowly activating K^+ current that is important for timely myocyte repolarization. Mutations in two human I_{ks} channel subunits, hKCNQ1 and hKCNE1, prolong APD and cause inherited cardiac arrhythmias known as LQTS (long QT syndrome) (Sanguinetti et al., 1996; Barhanin e al., 1996). We have found that KCNQ1 channels are present in increased density at the specialized zones of contacts of cardiac myocytes and SGN. An increased density of Na_v and KCNQ1 channels at the neuro-cardiac sympathetic junctions may have an important role in modulation of cardiac action potential by neuronal input.

Methods

Co-culture of SGNs and neonatal mouse ventricular myocytes.

SGNs were isolated from the cervical ganglia of newborn mouse pups by treating ganglia

with collagenase type 1A-S (Sigma-Aldrich) and trypsin T XI (Sigma-Aldrich) followed by trituration. Neurons were plated on coverslips coated with laminin (Sigma-Aldrich) for immunocytometry as described previously (Shcherbakova et al., 2007). Spontaneously beating neonatal cardiac myocytes were prepared from hearts of newborn mouse pups as described previously (Devic et al., 2001) and were added to already plated SGNs on the same day. After culturing for 24 h, co-cultures were treated with 1 µM cytosine arabinoside (Sigma-Aldrich) for 24 h to inhibit fibroblasts growth. Co-cultures were maintained in Leibovitz's L-15 medium supplemented with Nu serum (BD Biosciences), NGF (Invitrogen), and ITS liquid media supplement (Sigma-Aldrich). After cytosine arabinoside treatment, media were changed every 3 d as previously described.

Immunofluoresence microscopy.

Co-cultures were fixed by adding PBS (Mediatech, Inc.) containing 8% PFA directly to the culturing media to achieve a final PFA concentration of 4%. Cells were permeabilized with 1% BSA solution in PBS containing 0.2% Triton X-100. Cells were then stained with the desired antibody. The antibodies used were as follows: anti-tyrosine hydroxylase (mouse monoclonal; 1:800; Transduction Laboratories), anti-Ankyrin G and anti-KCNQ1 (1:800, rabbit polyclonal) were described earlier (Mohler et al.,2003); anti-pan Na_v (mouse monoclonal; 1:200; NeuroMab). The primary antibodies were detected with AlexaFluor594-conjugated goat anti–mouse IgG (1:1,000; Invitrogen) and AlexaFluor488 goat anti–rabbit IgG (1:1,000; Invitrogen). The slices for imaging were mounted with Vectashield mounting media (Vector Laboratories). The images were acquired at room temperature on an imaging microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc.) using a plan-Apochromat 63X 1.40 NA oil lens (Carl Zeiss MicroImaging, Inc.), a camera (RTE/CCD-1300-Y/HS; Roper Scientific), and IPLab software (BD Biosciences). Confocal images were acquired using a confocal laser-scanning microscope (LSM510; Carl Zeiss MicroImaging, Inc.) using Argon and He/Ne lasers and a plan-Apo 63X 1.4 NA or plan-Apo 100x 1.1 NA oil lenses, and images were analyzed by Volocity software (Improvision) and ImageJ.

Results

Scaffold protein ankyrin G serves to target and stabilize membrane proteins in cardiomyocytes. It localizes to critical domains in cardiac myocytes, such as T-tubule membranes and intercalated disks, which are specialized sites of contact between

cardiomyocytes that connect individual cardiac myocytes to work as a single functional organ or syncytium (Makara et al., 2018). At the intercalated disc, gap junctions electrically couple adjacent cells, acting as low resistance pathways to propagate action potentials between cardiomyocytes, i.e. as excitable domains in myocardium. Based on the localization of ankyrin G to critical sites in cardiac myocytes, we proposed that ankyrin G might be localized to another important compartment – junctions of cardiac myocytes with sympathetic neurons. We have tested this in co-cultures of cardiac myocytes and SGN by immunostaining with antibodies to ankyrin G and tyrosine hydroxylase. Indeed, we observed that ankyrin G is localized to the sites of contact of cardiac myocytes and SGN (Fig.1).

Ankyrin G is known to interact with Na_v channels and retain them at the excitable domains of membranes in neurons and cardiac myocytes (Pan et al., 2006; Hill et al., 2008; Rasband, 2011; Makara M.A. et al., 2014). Consistent with this, we have observed an increased density of Na_v channels using pan-Na_v channel antibodies (Fig.2 or Fig1.Supplement).

The excitable domains in neurons are organized by means of accumulation of Na_v channels responsible for initiation and propagation of action potential and K_v channels that shape the downstroke of the action potential (Hill et al., 2008; Rasband, 2011). In cardiac myocytes, repolarization is primarily achieved by I_{ks}, mediated by KCNQ1-KCNE1 and possibly other KCNQ1-KCNE combinations (Sanguinetti et al., 1996; Barhanin e al., 1996). It is well established that input if sympathetic nervous system increases the slow outward potassium ion current (I_{KS}) carried by KCNQ1-KCNE1 channel (Kass and Wang, 2000; Marx, 2002). Thus, we have tested localization of KCNQ1 channel to the neuro-cardiac junction. Indeed, we have found that KCNQ1 channels are present in increased density at the specialized zones of contacts of cardiac myocytes and SGN (Fig.3).

Discussion.

Localization and targeting of the cardiac ion channels are critically important for efficient action potential initialization and propagation through myocardium. In the present study we addressed localization of important cardiac ion channels and scaffold protein ankyrin G to the sites of sympathetic innervation of cardiac myocytes. We have found that ankyrin G localizes to the postsynaptic sites in co-culture of cardiac myocytes and SGN. Consistent with role of ankyrin G in targeting and clustering ion channels, we have found that Na_v channels are present in increased density at the specialized zones of contacts of cardiac

myocytes and SGN. In cardiac myocytes ankyrin G is involved in surface expression of $Na_v1.5$ channels and in its targeting to intercalated disks (Makara et al., 2014). Ankyrin-G cKO mice display bradycardia and arrhythmia associated with catecholaminergic stress (Makara et al., 2014). Taken together with our findings, this suggests that local modulation of Na_v channels by sympathetic input is important for proper transmission of the catecholaminergic signals to the heart.

KCNQ1 is unique potassium channel that have the capacity to form either channels that are voltage-dependent and require membrane depolarization for activation, or constitutively active channels (Abbott, 2014). KCNQ1 interacts with all five members of the KCNE family of single transmembrane domain ancillary or beta subunits, also called MinK-related peptides, MiRPs (Sanguinetti et al., 1996; Barhanin et al., 1996; McCrossan and Abbott, 2004). In human ventricular myocytes, KCNQ1-KCNE1 channels generate I_{Ks}, a slowly activating K^+ current. Delay in repolarization, caused by mutations in KCNQ1, can cause the potentially lethal cardiac arrhythmias. Human KCNQ1-KCNE1 channel heteromer in cardiac myocytes is part of a macromolecular complex formed by direct interaction with Yotiao, PKA, protein phosphatase1, phosphodiesterase 4D3 (PDE4D3), and adenylyl cyclase 9 (Marx et al.,, 2002; Terrenoire C. et al., 2009; Li J.Y. et al., 2009). We have not tested localization of other proteins comprising this signaling complex to the neuro-cardiac junctions. Further studies are warrantied to address the localization and dynamics of other proteins of this macromolecular complex.

It it worth noting that the localization of KCNQ1 and Na_v channels is not exclusive for the neuro-cardiac junctions: they are enriched at these sites, but they also expressed over the cardiac myocytes plasma membranes. This argues for compartmentalized regulation of these channels in the heart. For instance, it has been shown that there are two populations of K_v4 channels responsible for I_{to} current in cardiac myocytes, one localized to caveolae and the other localized to flat rafts. Interestingly, only the caveolae located population of K_v4 channels can be regulated by α1-AR stimulation and responsiveness of the K_v4 channels to catecholaminergic stimulation depend on scaffold protein AKAP100 (Alday et al., 2010).

Therefore, we observed that ankyrin G localized to the neuro-cardiac sympathetic junctions and Na_v and KCNQ1 channels are present in an increased density at that sites. Functional significance of localization of Na_v and KCNQ1 channels and ankyrin G to the neuro-cardiac sympathetic junctions is yet to be determined.

Acknowledgements: I am grateful to Dr. Brian K.Kobilka (Stanford University) for opportunity to conduct these experiments in his lab and Drs. Peter J. Mohler and Thomas Hund (The Ohio State University) for kindly providing anti-Ankyrin G and anti-KCNQ1 antibodies, helpful discussion and critical reading of the manuscript.

References:

Abbott, G. W. (2014). Biology of the KCNQ1 Potassium Channel. New Journal of Science Volume 2014, doi:10.1155/2014/237431.

Abbott, G. W., Goldstein, S. A. N., and Sesti, F. (2001). Do all voltage gated potassium channels use MiRPs? Circulation Research, 88:10, 981–983.

Alday, A, Urrutia, J, Gallego, M, Casis, O.(2010). α1-adrenoceptors regulate only the caveolae-located subpopulation of cardiac K(V)4 channels. Channels (Austin). May-Jun;4:3,168-78.

Armour, J.A., and Ardell, J.L. (1994). Neurocardiology. Oxford University Press,

New York, NY.

Barhanin, J., Lesage, F., Guillemare, E., Fink, M., Lazdunski, M., Romey, G. (1996). K(V)LQT1 and lsK (minK) proteins associate to form the I(Ks) cardiac potassium current.

Nature.7;384(6604):78-80.

Hill, A.S., Nishino, A., Nakajo, K., Zhang, G., Fineman, J.R., Selzer, M.E., Okamura, Y., Cooper, E.C. (2008). Ion channel clustering at the axon initial segment and node of Ranvier evolved sequentially in early chordates. PLoS Genet. 4:12:e1000317. doi: 10.1371/journal.pgen.1000317.

Kass, R. S., Wang, W. (2000). “Cardiac Electrophysiology from Cell to Bedside”, D. P. Zipes, J. Jalife, Eds. (Saunders, Philadelphia), chap. 11.

Li,]. Y., Chen L., Kass, R. S., Dessauer, C. W. (2012). The A-kinase anchoring protein Yotiao facilitates complex formation between adenylyl cyclase type 9 and the I_{Ks} potassium channel in heart. The Journal of Biological Chemistry, 287, 29815–29824.

Makara, M.A., Curran, J., Lubbers, E.R., Murphy, N.P., Little, S.C., Musa, H., Smith, S.A., Unudurthi, S.D., Rajaram, M.V.S., Janssen, P.M.L., Boyden, P.A., Bradley, E.A., Hund, T.J., Mohler, P.J.(2018). Novel Mechanistic Roles for Ankyrin-G in Cardiac Remodeling and Heart Failure. JACC Basic Transl Sci.12;3(5):675-689. doi: 10.1016/j.jacbts.2018.07.008.

Makara, M.A., Curran, J., Little, S.C., Musa, H., Polina, I., Smith, S.A., Wright, P.J., Unudurthi, S.D., Snyder, J., Bennett, V., Hund, T.J., Mohler, P.J. (2014). Ankyrin-G coordinates intercalated disc signaling platform to regulate cardiac excitability in vivo. Circ Res. 7;115(11):929-38. doi: 10.1161/CIRCRESAHA.115.305154.

Marx, S. O., Kurokawa, J., Reiken, S., Motoike, H., D’Armiento, J., Marks, A. R., Kass, R.S.(2002). Requirement of a macromolecular signaling complex for beta-adrenergic receptor modulation of the KCNQ1-KCNE1 potassium channel,” Science, 295:5554, 496–499.

McCrossan, Z.A. and Abbott, G. W. (2004) The MinK-related peptides. Neuropharmacology, 47:6, 787–821.

Mohler, P.J., Rivolta, I., Napolitano, C., LeMaillet, G., Lambert, S., Priori, S.G., Bennett, V.(2004). Nav1.5 E1053K mutation causing Brugada syndrome blocks binding to ankyrin-G and expression of Nav1.5 on the surface of cardiomyocytes. Proc Natl Acad Sci U S A. 14;101(50):17533-8.

Pan, Z., Kao, T., Horvath, Z., Lemos, J., Sul, J.Y, Cranstoun, S.D, Bennett, V, Scherer, S.S, Cooper, E.C. (2006). J Neurosci. A common ankyrin-G-based mechanism retains KCNQ and Na_V channels at electrically active domains of the axon. 8;26(10):2599-613.

Prando, V., Da Broi, F., Franzoso, M., Plazzo, A.P., Pianca, N., Francolini, M., Basso, C., Kay, M.W, Zaglia, T., Mongillo, M.(2018). Dynamics of neuroeffector coupling at cardiac sympathetic synapses. J Physiol. 596(11):2055-2075. doi: 10.1113/JP275693.

Rasband, M. N. Composition, assembly and maintenance of excitable membrane domains in myelinated axons.(2011). Semin Cell Dev Biol. 22(2): 178–184. doi:10.1016/j.semcdb.2010.09.010.

Sanguinetti, M.C., Curran, M.E., Zou, A., Shen, J., Spector, P.S., Atkinson, D.L., Keating, M.T. (1996). Coassembly of K(V)LQT1 and minK (IsK) proteins to form cardiac I(Ks) potassium channel. Nature.7;384(6604):80-3.

Shah, M., F.G. Akar, and G.F. Tomaselli. (2005). Molecular basis of arrhythmias. Circulation. 112:2517-29.

Shcherbakova, O.G, Hurt, C.M, Xiang, Y, Dell'Acqua, M.L, Zhang, Q, Tsien, R.W, Kobilka, B.K. (2007). Organization of beta-adrenoceptor signaling compartments by sympathetic innervation of cardiac myocytes. J Cell Biol. 12;176(4):521-33.

Terrenoire, C., Houslay, M. D., Baillie, G. S., Kass, R. S.(2009). The cardiac I_{Ks} potassium channel macromolecular complex includes the phosphodiesterase PDE4D3. The Journal of Biological Chemistry, 284:14, 9140–9146.

Figure legends.

Figure 1. Ankyrin G localizes to neuro-cardiac junctions.

A. Co-culture of cardiac myocytes and sympathetic neurons (DIV 5) immunostained with antibodies to Tyrosine Hydroxylase (red) and Ankyrin G (green). Confocal imaging, x100, single slice. B. Enlarged fragment of the image A, 3D reconstruction. C, D. Tyrosine Hydroxylase immunostaining (red channel of the images A and B correspondingly). F, G. Ankyrin G immunostaining (green channel of the images A and B correspondingly).

Figure 2. Na_v channels are enriched at the neuro-cardiac junctions.

A. Co-culture of cardiac myocytes and sympathetic neurons (DIV 5) immunostained with antibodies to Tyrosine Hydroxylase (green) and pan-Na_v (red). Epi-fluorescent image, x63. B. Tyrosine Hydroxylase immunostaining (green channel). C. Immunostaining for pan-Na_v antibodies (red channel).

Figure. 3. KCNQ1 channels are enriched at the neuro-cardiac junctions.

A. Co-culture of cardiac myocytes and sympathetic neurons (DIV 5) immunostained with antibodies to Tyrosine Hydroxylase (red) and KCNQ1 (green). Confocal imaging, x63, single slice. B. Enlarged fragment of the image A, 3D reconstruction. C,D. Tyrosine Hydroxylase immunostaining (red channel of images A and B correspondingly). F,G. KCNQ1 immunostaining (green channel of images A and B correspondingly).

Fig.1. Ankyrin G localizes to neuro-cardiac junctions.

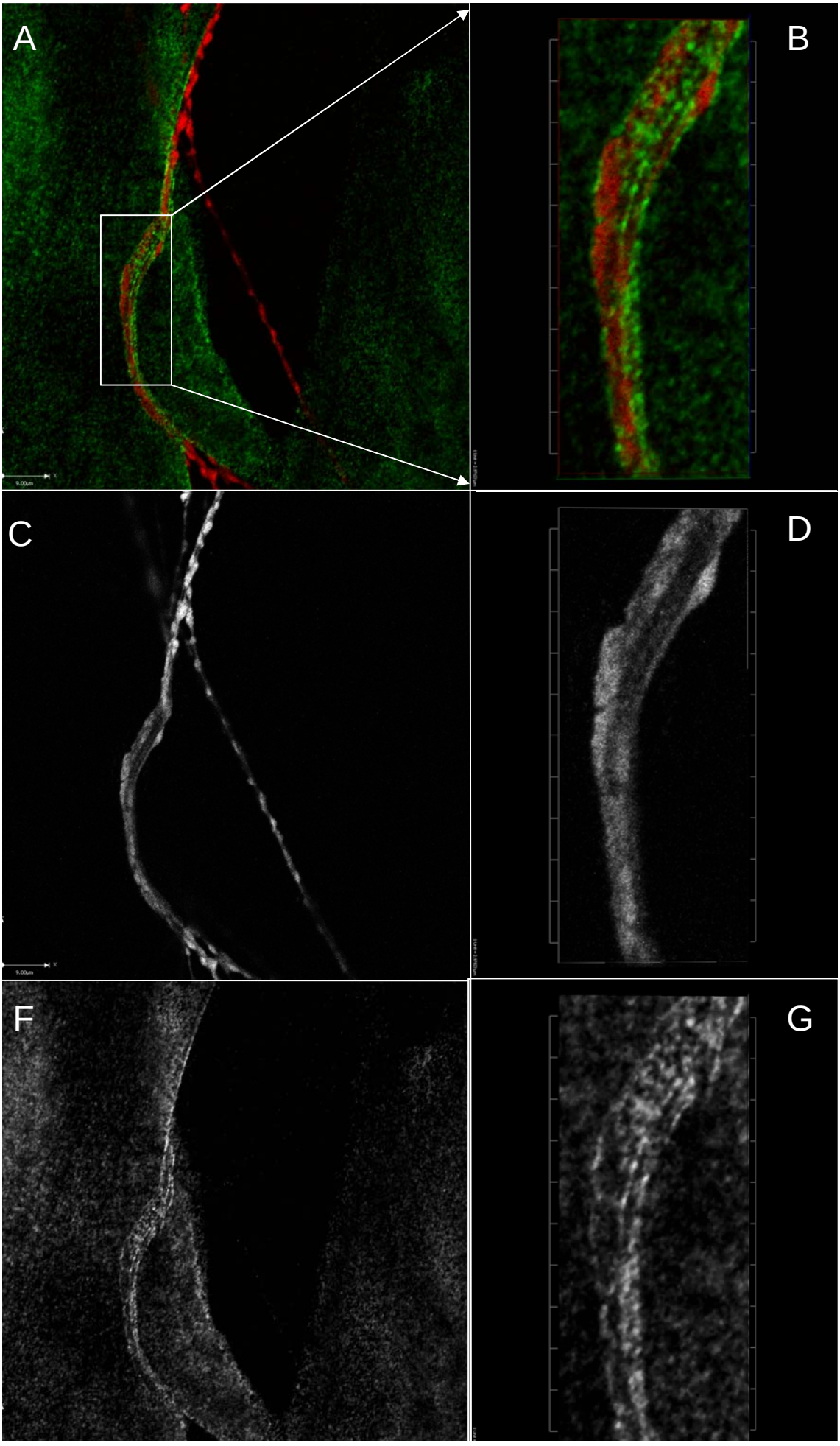

Fig.2: Na_V channels are enriched at the neuro-cardiac junctions.

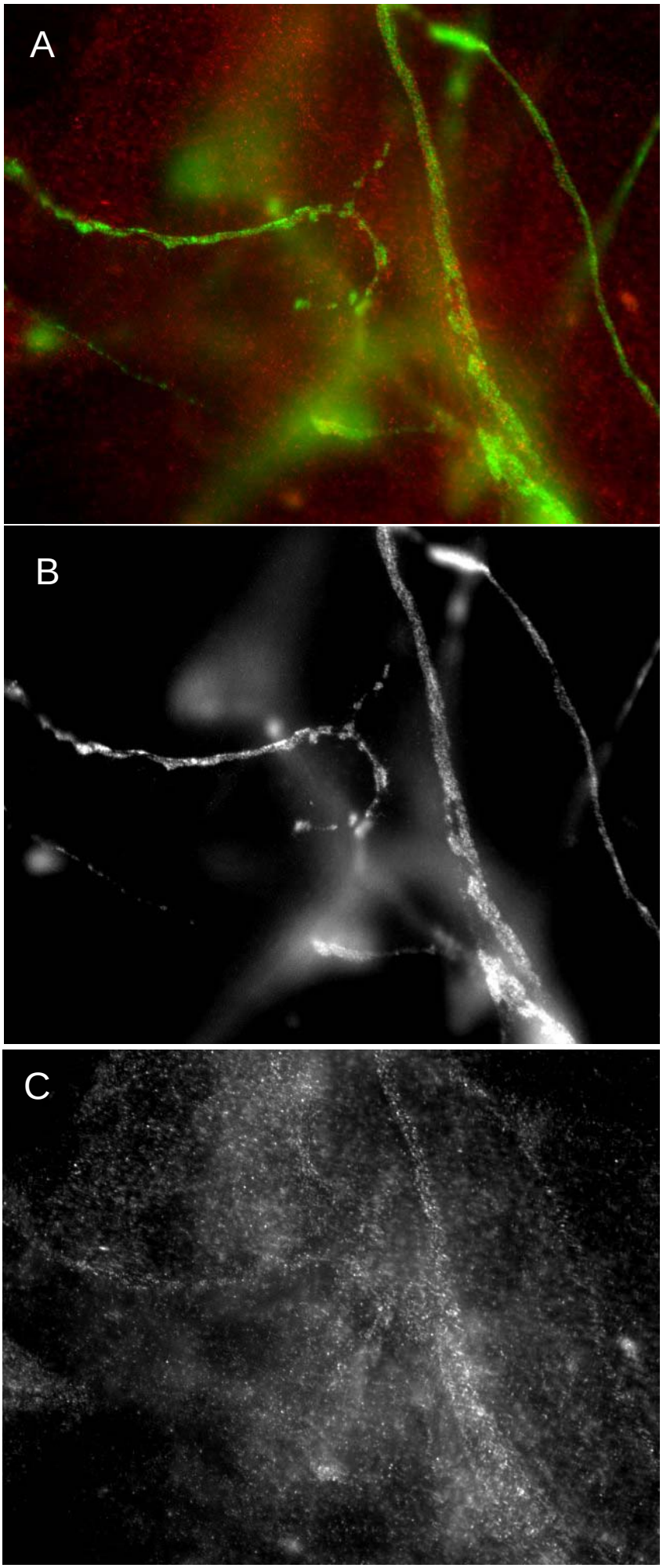

Fig.3. KCNQ1 channels are enriched at the neuro-cardiac junctions.