HHS Public Access

Author manuscript

Circ Res. Author manuscript; available in PMC 2023 July 22.

Published in final edited form as:

Circ Res. 2022 July 22; 131(3): 283–285. doi:10.1161/CIRCRESAHA.122.321167.

Na⁺, K⁺-Pump/Phospholemman Are Concentrated at Intercalated Discs for Conduction

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Subject Terms:

Basic Science Research; Ion Channels/Membrane Transport; Cell Biology/Structural Biology

Na^+,K^+-ATPase (NKA) and its phosphoprotein regulator phospholemman (PLM) critically modulates Na^+ and K^+ transports in cardiomyocytes.[1] Responding to adrenergic signaling, the PLM/NKA system regulates intracellular Ca^{2+} levels and excitation-contraction at cardiac T-tubules (TTs),[1] reducing triggered arrhythmias while preserving inotropy.[2] The coordinated trafficking and localization of NKA and PLM in cardiomyocytes remain incompletely understood, despite many studies on the possible role(s) of NKA and PLM in heart.[1]

We have generated and characterized multiple antibodies that identify PLM or NKA in a highly species-specific manner (Fig.1A–E). Fig. 1A shows the primary sequence of PLM in several species and epitopes of anti-PLM antibodies. Accordingly, the PLM monoclonal antibody, B8, and the polyclonal PLM-N-term antibody, specifically recognized dog PLM, but not rat, as a single protein band of ~15kDa by SDS-PAGE analyses of dog sarcolemmal membranes (*SL*), consistent with our previous reports in which PLM was purified and studied.[3] Meanwhile, the polyclonal antibody, PLM-C-term, detected PLM in both rat homogenates (*HM*) and membrane vesicles (*MV*) and dog heart preparations (Fig. 1A). In the case of NKA antibodies, Pan-α (to residues 762–775 of human α1 subunit) recognized both α1 and α2 subunits of NKA, while NKA-α1 (to residues 828–843) recognized α1, but not α2 subunit of human, dog, and rat NKA. Immunoblotting confirmed that anti-NKA-α1 and Pan-α were highly specific in detecting NKA in canine and rat cardiac samples as a single band at ~100kDa (Fig. 1A).

We performed confocal immunofluorescence assays in dog, rat, rabbit, and human heart tissue sections to determine the intracellular distribution of NKA and PLM. These five

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Conflict of Interest: None declared.

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antibodies all specifically stained dog cardiomyocytes (Fig. 1B, *upper panels*), with fluorescence signals that were more intense at intercalated discs (IDs) than TTs. Staining was eliminated by pre-incubation of antibodies with corresponding competing peptides (Fig. 1B), demonstrating the specificity for these antibodies. Fig. 1C shows that B8 and NKA-α1 signals highly overlapped, with intensity ratios between ID and TT at 3.06±0.5 and 2.88±0.5, respectively. Similar patterns of relative intensities were observed using PLM-C-term, NKA-α1, and Pan-α in rat, human, and rabbit cardiomyocytes (Fig. 1B, *lower panels*), confirming the findings in dog cardiomyocytes. Interestingly, B8 and PLM-N-term antibodies did not stain rat and human cardiomyocytes, confirming their high species-specificity in detecting PLM in dog cardiomyocytes, serving as a negative control. Furthermore, B8 and NKA-α1 stained TT of skeletal muscle myocytes, which do not develop IDs. Finally, NKA-α1 or PLM-N-term (Fig.1D, *green*) co-immunostained with three ID protein markers, (connexin 43, γ-catenin, or N-cadherin), confirming the co-localization of NKA and PLM to the ID.

To corroborate the steady-state localization of PLM at the ID, we applied an established experimental approach that tracks newly made proteins as they traffic to intracellular sites.[4] Adult rat cardiomyocytes were treated with recombinant adenovirus encoding the cDNA of canine PLM, and newly-synthesized dog PLM was specifically detected using species-specific antibody B8. Immunoblotting analyses confirmed the expression of newly-made dog PLM in rat cardiomyocytes (Fig.1Ea). By 30h post-infection, dog PLM was detected in TT, with more concentrated protein apparent at IDs (*arrows*, Fig.1Eb). B8 fluorescence intensity ratio of ID/TT=2.6±0.5, n=50 cardiomyocytes). Depolymerization of microtubules with nocodazole prevented newly-made dog PLM trafficking to ID (*Noc*, Fig.1Eb). NKA-α1 and PLM-C-term antibodies confirmed high concentrations of both proteins at ID of cardiomyocytes (Fig.1Ec). The localization pattern of newly made PLM paralleled its steady-state distribution in tissue, suggesting that PLM differentially traffics to, and accumulates at, two disparate subcellular sites.

If NKA is highly concentrated at IDs, one might expect its inhibition to lower ventricular conductivity. We performed optical mapping studies on rabbit hearts with the sequential addition of the specific NKA inhibitors ouabain or digoxin. Activation maps (Fig.1Fa,b summarized in c), at pacing cycle lengths from 260ms to 300ms, showed that both drugs significantly increased activation times (AT) in a dose-dependent manner, which correlated to a decreased conduction velocity. Action potential duration was also prolonged, consistent with the known effects of intracellular Ca^{2+} overload by NKA blockade.

In summary, we used immunological analyses of a series of epitopes on both PLM and NKA to demonstrate for the first time that the α1 subunit of NKA and PLM are each expressed at a roughly three-fold higher concentration in ID compared to TT in cardiomyocytes. Such unique distributions of NKA/PLM might provide differential control of Na^+ and K^+ concentration gradients and membrane potentials at two subdomains, exerting strong and direct effects locally at ID, thereby regulating membrane excitability and ion transports (e.g., through connexins or Na channels, which have higher concentration at ID than sarcolemma[5]) for cardiac conduction. Mechanisms of PLM and NKA protein dual pathways trafficking to TT and ID, interaction with protein complexes (and beyond PLM modulation

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of NKA), and associated effects of PLM regulation by adrenergic signaling, provide important new insights into the biochemistry and physiology of cardiac contraction and conduction.

Acknowledgements

We thank Drs. Steve Cala, James E. Tisdale, and Rohan Dharmakumar for helpful discussions. We also thank Nicole Courtney for technical supports.

Sources of Funding

This study was supported in part by American Heart Association Grant #18TPA34170284 /ZC/2018, the Dr. Charles Fisch Cardiovascular Research Award endowed by Dr. Suzanne B. Knoebel of the Krannert Cardiovascular Research Center, NIH Grants R01HL139829, OT2OD028183-01, the Burns & Allen Chair in Cardiology Research, Cedars-Sinai Medical Center, Los Angeles, California, USA, and the Postdoctoral Research Abroad Program, Ministry of Science and Technology, Republic of China (Taiwan).

Data Availability

The methods, data, and materials are available upon request. The use of animals in the study was approved by the IACUC of Indiana University School of Medicine and the Methodist Research Institute, Indianapolis, Indiana and the IACUC of Cedars-Sinai Medical Center, Los Angeles, California and conformed to the NIH Guide for the care and use of laboratory animals.

Abbreviations:

ID	intercalated discs
NKA	Na^+, K^+-ATPase
PLM	phospholemman
TTs	T-tubules

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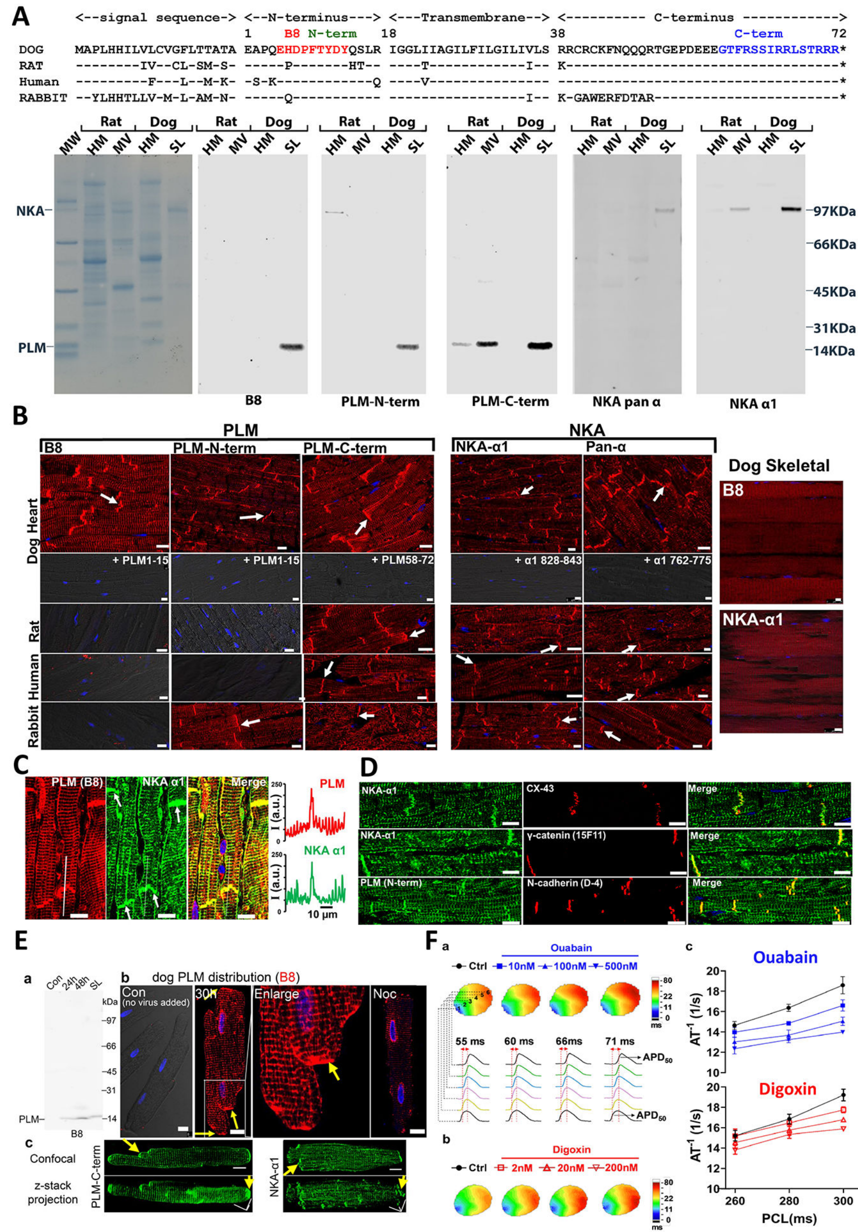

Figure 1:

A.B., Immunoblots (**A**) and confocal immunofluorescence images (**B**) showing detection of PLM and α1 subunit of NKA in heart and skeletal muscles, with affinity purified antibodies, including anti-PLM antibodies: B8, PLM-N-term, and PLM-C-term (*top* shows antibodies binding epitopes); and anti-NKA antibodies, NKA-α1 and Pan-α. Antibodies were visualized by protein-A-Alexa-647 fluorescence dye. 10μg of rat or dog heart homogenates (*HM*), membrane vesicles (*MV*), and sarcolemmal membranes (*SL*) were used. In control, antibodies were added together with blocking peptides. **C.D.,** Confocal immunofluorescence images showing co-staining of **C,** PLM (*red*) and α1 subunit of NKA (*green*) (with plot of intensity profiles along the white line); or **D,** connexin-43, γ-catenin, or N-cadherin (*red*) and NKA-α1 or PLM-N-term (*green*) in dog ventricular sections. Similar

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results were obtained in 5 animals (A–D). **E.** Immunoblots (a) and immunofluorescence images showing expression of dog PLM in rat cardiomyocytes, probed with B8 (b), and PLM-C-term and NKA-α1 (c). Noc, 10μM nocodazole was added 6h post-infection. (>30 cardiomyocytes at each condition). Arrows indicate IDs in B-E. Blue indicates DAPI. Bar is 10μm. **F.** Optical mapping of Langendorff perfused rabbit hearts after digitalis infusion. a,b. Sample activation maps of right ventricle pacing at 300ms pacing cycle length (PCL). Bottom plots show action potentials at each point . c. Inverse activation time (AT^{-1}) at each PCL (n=5 rabbits for ouabain, n=4 for digoxin). Linear mixed effects models show that AT^{-1} decreases significantly as ouabain or digoxin dose increases (p=1.26E-07 or p=5.24E-05, respectively). All figures were selected to highlight the typical results of the study.