Investigating the co-assembly and palmitoylation of Kv4.3 splice variants

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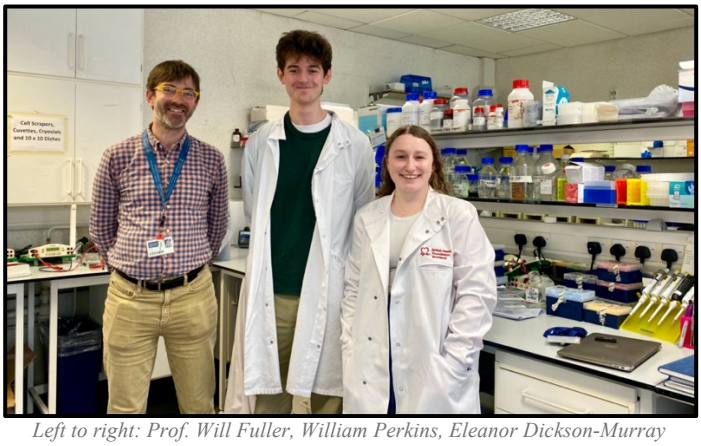


Left to right: Prof. Will Fuller, William Perkins, Eleanor Dickson-Murray

Introduction

The cardiac action potential is a transient, reversible change in transmembrane voltage that is essential for orchestrating the rhythmic contraction and relaxation of the heart. The principal ion current contributing to phase one of the cardiac action potential is the cardiac transient outward potassium current (I_{to}), which is mediated by the voltage-gated potassium channel Kv4.3. Importantly, Kv4.3 function and expression are markedly downregulated in heart diseases (e.g., hypertrophy, heart failure, and myocardial infarction) [1]. Kv4.3 forms a homotetrameric channel and its monomers naturally exist as two alternatively spliced isoforms differing by a C-terminal, 19 amino acid region [2]. The splice variants co-exist in the mature myocardium, but their expression is differentially altered in cases of heart failure. In non-failing hearts, mRNA expression of the variants is similar, but in failing hearts the long variant (Kv4.3-L) is upregulated ~33% and the short variant (Kv4.3-S) is downregulated ~75% [3]. Kv4.3 can be palmitoylated, the post-translational addition of a fatty acid, at two cysteine residues in its disordered C-terminal tail (Fuller Lab, unpublished results). In transiently transfected cells, palmitoylation enhances the magnitude of Kv4.3-mediated currents, but the long variant is ~2.5-fold less palmitoylated than the short (Fuller Lab, unpublished results). This implies the dominance of the long variant over the short in heart disease may contribute to the downregulated Kv4.3-mediated currents observed in failing hearts.

Aims of the project

Experiments to date have focused on studying Kv4.3 splice variants separately. This project will investigate biochemical consequences of co-expressing the short and long splice variants of Kv4.3. Specifically, to: (i) establish whether the long and short splice variants of Kv4.3 physically co-assemble; and (ii) assess whether the established difference in palmitoylation between the short and long splice variants of Kv4.3 persist when they are co-expressed. This project also explores how alternative splicing can act as a regulator of protein palmitoylation in the case of Kv4.3.

Methods used

Plasmid DNA purification: QiaPrep Spin Miniprep kits were used to isolate plasmid DNA as per manufacturer's instructions. Final DNA concentration was measure spectrophotometrically and samples were stored at -20°C. Samples were sent to Eurofins Genomics for confirmatory sequencing.

Mammalian cell culture and transfection: Human embryonic kidney cells (HEK-293) were used in all experiments. Cells were cultured at 37°C and 5% CO_2 atmosphere and passaged 2-3 times/week in complete culture media (DMEM, 10% foetal bovine serum, 1% penicillin-streptomycin). Plated cells were grown to approximately 80% confluency, transiently transfected using Lipofectamine 2000, and incubated for 18-24h before harvesting.

SDS-PAGE and Western blotting: Samples were resuspended in 2X Laemmli sample buffer (SB), heated at 60°C for 10min and separated on 6-20% Tris-Glycine gradient gels. Gels were transferred onto 0.2µm PVDF membrane for semi-dry transfer using a Bio-Rad Transblot Turbo. Images were acquired using the LI-COR Odyssey and bands quantified using Image Studio.

Acyl-resin assisted capture (Acyl-RAC): Palmitoylated proteins were purified using Acyl-RAC. Free thiol (-SH) groups are blocked using methyl methanethiosulfonate (MMTS) and subsequently thioester bonds are specifically cleaved by hydroxylamine, whereupon a thiol-reactive resin (thoiopropyl sepharose) selectively captures exposed thiols from palmitoylated proteins [4].

Co-immunoprecipitation (Co-IP): Previously transfected cells were lysed for 30min in lysis buffer (1:1000 protease inhibitor cocktail, 1% Triton-X in PBS). Lysates were centrifuged at 17,000g for 5 minutes at 4°C and insoluble material was discarded. GFP-tagged proteins were immunoprecipitated from the soluble cell lysate using magnetic GFP-trap agarose, and beads were washed 3 x 1mL in wash buffer (0.25% Triton-X in PBS).

Results

(i) Do Kv4.3 long and short splice variants interact?

GFP-trap co-immunoprecipitation was performed (n=3) to investigate protein-protein interactions between Kv4.3S/L. HEK-293 cells were co-transfected with untagged Kv4.3S/L and YFP-tagged Kv4.3S/L in a 20:80 ratio (tagged:untagged), empirically determined to yield approximately equal expression levels of the variants. Figure 1 supports an interaction between the splice variants, as YFP-tagged Kv4.3S/L clearly co-purifies the untagged transfection partner.

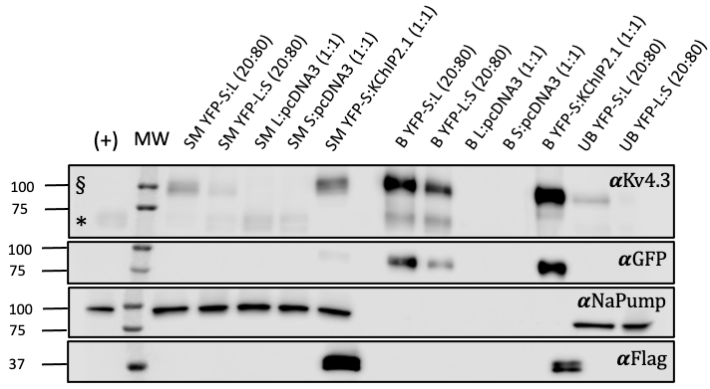


Figure 1. Investigating co-assembly of Kv4.3 splice variants by GFP-trap co-immunoprecipitation (n=3). HEK-293 cells were transiently transfected with YFP-tagged (100kDa, §) and untagged (70kDa, *) Kv4.3S/L (20:80 ratio, tagged:untagged), alongside a negative control, the empty vector pcDNA3, and a positive control for Flag-tagged KChIP2.1, a known Kv4.3 interactor. The starting material (SM), bead fraction (B) and unbound fraction (UB) are denoted. SDS-PAGE and western blotting was performed to visualise proteins with membranes probed for Kv4.3, GFP, Na+ Pump (ubiquitous membrane protein), and Flag (detecting KChIP2.1). Immunoprecipitating YFP-S co-purifies untagged Kv4.3L (lane B YFP-S:L) and immunoprecipitating YFP-L co-purifies untagged Kv4.3S (lane B YFP-L:S).

(ii) Does co-expressing Kv4.3S/L alter levels of palmitoylation?
HEK-293 cells were co-transfected with different ratios of tagged and untagged Kv4.3S/L and acyl-RAC assays were performed (n=6) to determine the levels of protein palmitoylation. Figure 2 reveals that Kv4.3S and L can be palmitoylated in the presence of each other.

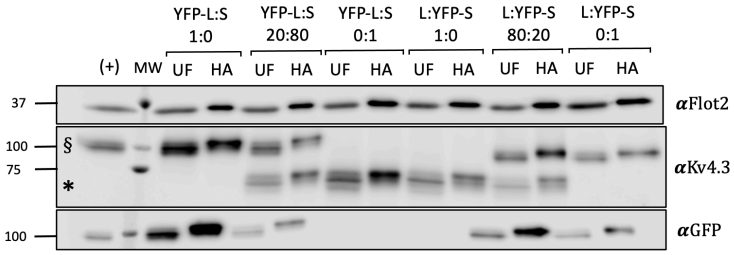
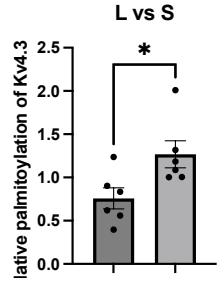


Figure 2. Effect of co-expressing Kv4.3 splice variants on palmitoylation state (n=6). Acyl-RAC of HEK-293 cells transfected with YFP-tagged (100kDa, §) and untagged (70kDa, *) Kv4.3S/L in various ratios. SDS-PAGE and western blotting was performed to visualise proteins; the unfractionated sample (UF, all proteins in lysate) and hydroxylamine-treated sample (HA, bead fraction containing palmitoylated proteins) are denoted. Membranes were probed for Flot2 (a reliably palmitoylated protein, relatively enriched in HA fraction), Kv4.3 (protein of interest), and GFP (transfection control).

Western blots were subsequently quantified by normalising the UF/HA ratio to the experimental mean of the day. Experimental groups were compared using t-tests or one-way ANOVA with post-hoc Sidak's multiple comparisons test.



Previous experimentation has established that the Kv4.3L is ~2.5-3 times less palmitoylated than Kv4.3S. A statistically significant difference in palmitoylation levels ($p<0.05$) between the variants was similarly observed here, albeit slightly smaller in magnitude.

Figure 3. Difference in palmitoylation between splice variants. A t-test was performed comparing the normalised relative palmitoylation of Kv4.3S/L in isolation, n=6, *: $p<0.05$, t-test.

It was hypothesised that Kv4.3L is less palmitoylated than Kv4.3S either: (i) because it is a poor substrate for the palmitoylating enzyme; or (ii) because it exists in a different sub-cellular compartment from the palmitoylating enzyme. We investigated whether mixed Kv4.3 oligomers formed of Kv4.3L and Kv4.3S retained the difference in palmitoylation between the splice variants (figure 4).

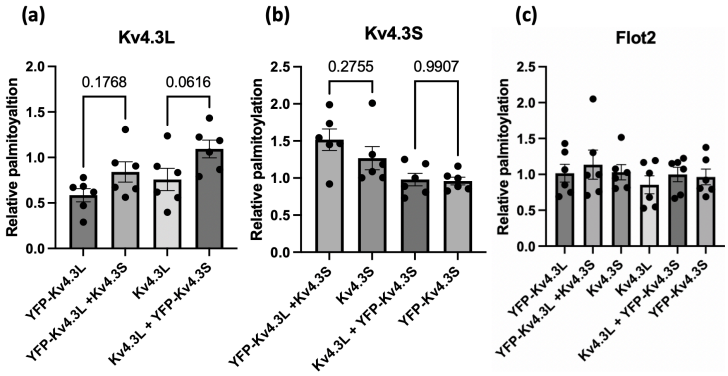


Figure 4. Quantification of Acyl-RAC western blots. a) Palmitoylation of Kv4.3L in the absence and presence of Kv4.3S. **b)** Palmitoylation of Kv4.3S in the absence and presence of Kv4.3L**. c)** Flot2 palmitoylation relative to expression. No statistically significant differences were observed.

It is evident that Kv4.3L becomes slightly more palmitoylated when Kv4.3S is expressed, but this is not a statistically significant effect for n=6 (figure 4a). Further, palmitoylation of Kv4.3S appears unchanged in the presence of Kv4.3L (figure 4b). This suggests Kv4.3L remains a poor substrate for the palmitoylating enzyme regardless Kv4.3S expression. Flot2 expression appears consistent across conditions (figure 4c), and Kv4.3 expression levels were appropriately titrated to be comparable (figure 5).

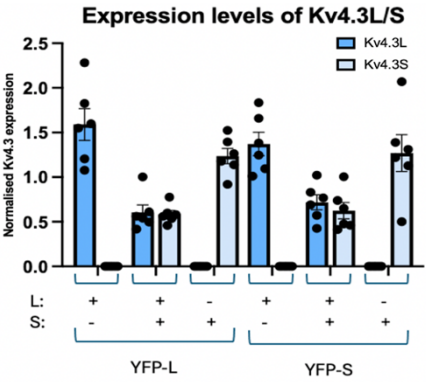


Figure 5. Expression levels of Kv4.3S/L. The expression of Kv4.3S/L have been closely matched in the double transfection, confirming the 80:20 ratio of constructs in the transfection mix was appropriate.

Future directions

It would be prudent to explore whether Kv4.3-L is less palmitoylated than Kv4.3-S due to reduced cell surface trafficking, limiting subsequent exposure to the palmitoylating enzyme. The established co-assembly of the variants could lead to Kv4.3S-assisted trafficking of Kv4.3L to the cell surface. Membrane-impermeable biotinylation reagents could be used to label and purify surface membrane resident proteins to evaluate trafficking and cell surface delivery of Kv4.3S/L splice variants when expressed in isolation or together. Further, experimentation was conducted in HEK-293 cells, which is not the native physiological environment of Kv4.3. Thus, these experiments could be repeated in cardiac cells.

Value of studentship

To the student: This project has enabled me to develop my confidence in the laboratory, fostering numerous technical skills and essential lab techniques which will be excellent preparation for my future academic pursuits. Additionally, I have developed many invaluable transferrable skills including organisation, problem solving, and communication through presenting my findings in a lab meeting. Further, working on an independent project within an active lab has strengthened my desire to pursue research. I am thrilled to have contributed towards our understanding of Kv4.3 given its connections to disease and strong translational potential.

To the lab: "This project is the first to evaluate the biochemical behaviour of Kv4.3 splice variants when they are expressed together rather than in isolation. As such this sets the foundation for future studies in my lab to understand the behaviour of mixed Kv4.3 oligomers. The data are high quality, and unequivocally show that mixed Kv4.3 oligomers form (Fig. 1) and offer hints that the presence of Kv4.3S may modify how the cell handles Kv4.3L (Fig. 4). Will's input has therefore meaningfully advanced our current Kv4.3-centred research programme." – Prof. Fuller

Acknowledgements

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