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Greasing The Wheels Or A Spanner In The Works? Regulation Of The Cardiac Sodium Pump By Palmitoylation

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Biographical notes

JH: Jacquie obtained a BSc in Anatomical Sciences from University Dundee, where she remained for a PhD investigating the role of the anterior gradient proteins in hormone related cancers. Since 2008 her research has focused on the cardiac accessory protein of the sodium pump, phospholemman: the proteins it interacts with and the post-translational modifications

that influence it.

KJW: Krzys obtained an MSc in biology focused on biochemistry, and PhD in molecular biology both from A. Mickiewicz University in Poznan, Poland. In his current postdoctoral research position he is studying compartmentalization and trafficking proteins essential for homeostasis and signaling in cardiac myocytes.

FP: Fiona received an undergraduate degree in pharmacology from the University of Dundee in 2015, during which she undertook a final year project in the Fuller lab, investigating the palmitoylation of the cardiac sodium-calcium exchanger. She is now continuing in this lab for her PhD, now concentrating upon the cardiac palmitoyl transferase, DHHC5.

LBT: Lindsay obtained his BSc in Genetics and PhD in Structural Biology and Biochemistry at the University of Edinburgh, where his research focused on structure-based drug design. Following postdoctoral posts at the University of Dundee involving structural and cell biology, he now works at the University of St Andrews investigating drug interactions in parasites.

NJF: Niall obtained a BSc in Biochemistry from the University of Glasgow where he stayed to do his PhD with Prof. Richard Cogdell FRS. Since then, he has been working independently on the structure and function of mammalian membrane proteins, and is currently focused on developing molecular modulators of protein palmitoylation for therapeutic gain.

WF: Will gained his BA in Pharmacology from University of Cambridge and remained in the Department of Pharmacology in Cambridge to study for a PhD. After postdoctoral research at King's College London he moved to Dundee in 2006 to establish a lab with interest in protein-protein interactions and post-translational modifications in cardiac muscle. He relocated his research programme to ICAMS in Glasgow in 2017.

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Greasing The Wheels Or A Spanner In The Works? Regulation Of The Cardiac Sodium Pump By Palmitoylation

The ubiquitous sodium / potassium ATPase (Na pump) is the most abundant primary active transporter at the cell surface of multiple cell types, including ventricular myocytes in the heart. The activity of the Na pump establishes transmembrane ion gradients that control numerous events at the cell surface, positioning it as a key regulator of the contractile and metabolic state of the myocardium. Defects in Na pump activity and regulation elevate intracellular Na in cardiac muscle, playing a causal role in the development of cardiac hypertrophy, diastolic dysfunction, arrhythmias and heart failure. Palmitoylation is the reversible conjugation of the fatty acid palmitate to specific protein cysteine residues; all subunits of the cardiac Na pump are palmitoylated. Palmitoylation of the pump's accessory subunit phospholemman by the cell surface palmitoyl acyl transferase DHHC5 leads to pump inhibition, possibly by altering the relationship between the pump catalytic α subunit and specifically bound membrane lipids. In this review we discuss the functional impact of phospholemman palmitoylation on the cardiac Na pump and the molecular basis of recognition of phospholemman by its palmitoylating enzyme DHHC5, as well as effects of palmitoylation on Na pump cell surface abundance in the cardiac muscle. We also highlight the numerous unanswered questions regarding the cellular control of this fundamentally important regulatory process.

Keywords: acylation; DHHC; palmitoyl acyl transferase, thioesterase; post-translational modification; P-type ATPase; ion transport; phospholemman

1. Introduction

The transmembrane sodium / potassium pump (Na pump) couples the hydrolysis of ATP to the cellular export of 3 sodium (Na) ions and the import of 2 potassium (K) ions against their electrochemical gradients, and operates ubiquitously in eukaryotic cells. As the most abundant primary active transporter at the cell surface in most cells and tissues (including cardiac myocytes), its activity is a key regulator of transmembrane ion gradients, secondary active transport, membrane potential, and consequently numerous

events at the cell surface. Acute and chronic changes in pump activity have profound effects on cell and organ physiology.

Multiple regulatory pathways converge on the cardiac pump. Pathways involving phosphorylation and oxidation are undoubtedly functionally important, but have been described and discussed in detail elsewhere (Rasmussen *et al.*, 2010, Fuller *et al.*, 2013, Liu *et al.*, 2013, Pavlovic *et al.*, 2013a, Bogdanova *et al.*, 2016). This review therefore focuses on a pathway of emerging importance in numerous aspects of membrane transport: the impact of fatty acylation of specific cysteine residues (palmitoylation) in the various constituent subunits of the Na pump on its activity in cardiac muscle. As the only reversible lipid modification, palmitoylation facilitates the dynamic regulation of the relationship between integral membrane proteins and their lipid environment. In order to appreciate the mechanistic impact of palmitoylation on the cardiac Na pump, we will first consider the quaternary structure of the pump, the (patho)physiological importance of pump regulation, and the regulatory milieu to which the pump is exposed (with particular emphasis on regulatory lipid interactions). Before that we will explore palmitoylation broadly as a reversible posttranslational modification.

2. Palmitoylation

Palmitoylation is the reversible conjugation of the fatty acid palmitate to specific cysteine thiol groups via a thioester bond. Catalyzed by integral membrane DHHC-containing palmitoyl acyl transferase enzymes, and reversed by thioesterases, this dynamic post-translational modification occurs throughout the secretory pathway. The hydrophobicity of the 16 carbon fatty acid palmitate that is attached to proteins is such that it must be accommodated within the hydrophobic core of a lipid bilayer, which

means that palmitoylation reversibly anchors proteins to membranes (Salaun *et al.*, 2010, Chamberlain and Shipston, 2015). The attachment of myristoyl or prenyl lipid groups to proteins achieves only weak or transient membrane association, with stable membrane attachment requiring double lipidation (Shahinian and Silvius, 1995). In contrast we find that even a single palmitoylation event is sufficient to act as a stable membrane anchor (Reilly *et al.*, 2015). The fact that the palmitoylating DHHC enzymes are themselves integral membrane proteins probably creates a requirement for most substrates to possess some intrinsic affinity for membranes – either through the presence of integral membrane domains, other lipid anchors (Hancock *et al.*, 1989), or other membrane-interacting sequences (Greaves *et al.*, 2008, Greaves *et al.*, 2009, Plain *et al.*, 2017). Thus, palmitoylation can both reversibly recruit soluble proteins to the bilayer functionally compartmentalizing them, and significantly restructure intracellular loops of transmembrane proteins, leading to functional changes in, for example, ion transporters (Shipston, 2011).

2.1 Palmitoylation dynamics: tools and pitfalls

Whilst the palmitoylation of some proteins is essentially static, it has long been appreciated that the palmitoylation of some proteins turns over quickly, particularly heterotrimeric and small G proteins (Magee *et al.*, 1987, Mumby *et al.*, 1994, Wedegaertner and Bourne, 1994). In recent years unbiased proteomic screens have found that a wide-range of palmitoylated proteins (including ion transporters and their regulators) rapidly cycle between palmitoylated and depalmitoylated states (Kang *et al.*, 2008, Martin *et al.*, 2012). Hence for a significant subset of the cellular palmitome, the turnover of palmitoylation is

sufficiently rapid to mediate acute physiological responses to hormonal and environmental challenges. Indeed, the list of dynamically palmitoylated proteins grows ever longer.

Although the experimental tools to investigate depalmitoylation kinetics (by targeting thioesterases) are relatively specific, the most commonly-used tool to investigate palmitoylation kinetics (targeting DHHC-PATs) is extremely blunt. Thioesterases identified to date are all members of the α/β -hydrolase family of serine hydrolases (Long and Cravatt, 2011, Lord *et al.*, 2013). The non-selective serine hydrolase inhibitor hexadecyl fluorophosphonate (HDFP) largely abolishes palmitoylation turnover by blocking all depalmitoylating enzymes (Martin *et al.*, 2012), while specific, selective and *in vivo*-active inhibitors of acyl protein thioesterases 1 and 2 (APT1, APT2) have been described (Adibekian *et al.*, 2012). The commercially-available β -lactone compound Palmostatin B, which was originally developed as a specific inhibitor of APT1 (Dekker *et al.*, 2010, Rusch *et al.*, 2011), has recently been reported to inhibit the activity of other α/β -hydrolases, including ABHD17 proteins, which depalmitoylate N-ras (Lin and Conibear, 2015). In contrast only a single reagent, 2-bromopalmitate (2-BP), is commonly used to pharmacologically manipulate the activity of DHHC-PATs, usually at high μ M concentrations. Although 2-BP is undoubtedly an effective inhibitor of some DHHC-PATs, studies using a 'clickable' analogue of 2-BP suggest it is extremely promiscuous in its reactivity (Davda *et al.*, 2013). ω -azido 2-BP fails to react with some DHHC-PAT isoforms, but labels 450 protein targets in intact cells, including palmitoylated proteins, transporters, channels and the α/β -hydrolase ABHD16A. Clearly the palmitoylation field urgently needs more specific DHHC-PAT inhibitors to interrogate cellular events with greater

certainty, and considerable caution should be used when considering any phenotypic consequences of 2-BP application. Indeed, in the absence of accompanying supportive genetic evidence (e.g. candidate cysteine mutagenesis, DHHC-PAT knockout or silencing), an effect of 2-BP alone should not be taken as evidence of a role for palmitoylation in regulating a particular cellular event.

2.2 Palmitoylation and the regulation of cardiac ion transporters

The Na pump is one of numerous ion transporters regulated by palmitoylation in cardiac muscle. Indeed, trans-sarcolemmal fluxes of Na, Ca and K ions are all influenced by palmitoylation of the transporters and channels involved. The cardiac sodium/calcium exchanger NCX1 is palmitoylated at a single cysteine in its large regulatory intracellular loop, which sensitizes it to inhibition by its integral ‘exchange inhibitory peptide’ (Reilly *et al.*, 2015, Fuller *et al.*, 2016). Palmitoylation of cysteines in the II-III linker of voltage-gated channel sodium SCN5a, which is responsible for the voltage activated Na current that initiates the cardiac action potential, increases channel availability and hence cardiac excitability (Pei *et al.*, 2016). The regulatory β_{2a} subunit of the cardiac L-type Ca channel is palmitoylated shortly after being translated; β_{2a} palmitoylation enhances L-type Ca channel activity (Chien *et al.*, 1996, Chien *et al.*, 1998). Several K channels and their regulatory subunits are also palmitoylated: for example surface expression of KCN5A, responsible for Kv1.5, is regulated by palmitoylation (Zhang *et al.*, 2007), and the ability of Kv4 family regulatory KChIP subunits to promote assembly and surface expression of mature channels relies on their palmitoylation (Takimoto *et al.*, 2002). Many aspects of excitation-contraction coupling, Ca handling and contractility, and the properties of the cardiac action potential are thus regulated by

palmitoylation. The importance of these events for the physiological control of cardiac function, and their relevance to defects in the mechanical and electrical behavior of the heart in disease are gradually becoming more widely appreciated.

3. The Cardiac Sodium Pump

3.1 Quarternary Structure of the pump: Cardiac Subunit Composition

The Na pump is a multi subunit enzyme, with the mature enzyme composed of two obligate subunits (α , β), which may be joined by a third regulatory subunit (FXYP).

The α subunit is the catalytic core of the enzyme, containing the binding sites for sodium, potassium and ATP as well as cardiotonic steroids such as ouabain. In order for the α subunit to traffic through the secretory pathway to the plasma membrane it must assemble with a β subunit in the endoplasmic reticulum (Geering, 1991, Horisberger *et al.*, 1991). The third member of the complex, which is called the FXYP protein after its conserved extracellular phenylalanine-X-tyrosine-aspartate motif (Sweadner and Rael, 2000) is not required for assembly of the mature pump, but does regulate its kinetic properties and/or substrate affinities.

There are four isoforms of the α subunit, three isoforms of β , and seven FXYP proteins in mammalian genomes. With respect to the catalytic subunit, $\alpha 1$, $\alpha 2$ and $\alpha 3$ are all detected in cardiac muscle (McDonough *et al.*, 1996, James *et al.*, 1999, Bossuyt *et al.*, 2005, Tulloch *et al.*, 2011, Wypijewski *et al.*, 2013), although most investigations have focused on $\alpha 1$ and $\alpha 2$ containing pumps, which are both functionally different and distributed differently in cardiac myocytes (discussed below). We find the $\alpha 3$ subunit associated with FXYP1 (phospholemman, PLM, which is myocyte-specific) in cardiac muscle, which implies that the $\alpha 3$ subunit detected in the heart is derived from

myocytes rather than a non-myocyte pool (Wypijewski *et al.*, 2013). However, the functional contribution of $\alpha 3$ to cardiomyocyte Na homeostasis is small under physiological conditions, although it may contribute to Na mishandling in cardiac pathologies (Semb *et al.*, 1998).

Until recently the principal β subunit in cardiac muscle was thought to be $\beta 1$ (although we have observed the $\beta 3$ subunit using proteomics to identify pump subunits prepared from rat ventricular myocytes (Tulloch *et al.*, 2011, Wypijewski *et al.*, 2013)). However, recent work suggests $\beta 2$ is also a major cardiac subunit (discussed below (Habeck *et al.*, 2016)). The principal cardiac FXYD protein is phospholemman (PLM). FXYD5 (RIC) has also been found in homogenates from whole hearts (Lubarski *et al.*, 2005), but whether this derives from a myocyte or non-myocyte population remains to be determined.

3.2 Importance of Pump Regulation in Cardiac Muscle

The Na pump supports normal electrical activity as well as establishing and maintaining ion gradients in all excitable tissues. In cardiac muscle, the Na gradient established by the pump drives numerous ion exchange and transport processes critical for normal cardiac function. Na-dependent membrane transporters include those responsible for the movement of other ions (e.g. Na/Ca exchanger (NCX), Na/H exchanger and Na-HCO₃ cotransporter (Mullins, 1981)), as well as those moving substrates (glucose, mannose, creatine, succinate and amino acids (Molitoris and Kinne, 1987)).

3.2.1 Relationship between Na pump and NCX

By controlling intracellular Na, the pump regulates NCX and hence cardiac contractility, because NCX uses the Na gradient established by the pump to remove Ca

from the cytosol and hence relax cardiac muscle. Alterations in NCX activity directly influence the rate of Ca removal from the cytoplasm and hence diastolic function. Moreover since NCX and SERCA essentially compete to remove Ca, alterations in NCX activity also indirectly influence systolic function, by changing the sarcoplasmic reticulum (SR) calcium content. Ultimately therefore changes in Na pump activity change cardiac contractility, and have the potential to be the driving force behind misregulation of Ca handling, which is a primary cause of contractile abnormalities and heart failure (Boguslavskyi *et al.*, 2014).

Experiments using detubulation (physical uncoupling of t-tubule membranes from the peripheral sarcolemma) indicate that in murine ventricular myocytes approximately 70% of $\alpha 2$ and 40% of $\alpha 1$ subunit mediated pump activities reside in the t-tubules. This is despite the fact that the t-tubules represent only 30% of total myocyte surface area (Berry *et al.*, 2007). A similar functional concentration of $\alpha 2$ activity in t-tubules (Despa *et al.*, 2003) and isoform distribution has been reported for the rat (Swift *et al.*, 2007). Both $\alpha 1$ and $\alpha 2$ are functionally coupled to NCX in ventricular myocytes (Dostanic *et al.*, 2004), and hence both subunits can indirectly control Ca handling and contractility through their influence on NCX. However when $\alpha 1$ - or $\alpha 2$ -containing pumps are individually blocked to give similar rises in intracellular sodium, only $\alpha 2$ blockade influences the amplitude of the calcium transient, suggesting $\alpha 2$ containing pumps are the principal regulators of NCX and hence Ca handling (Despa *et al.*, 2012). In support of this concept, transgenic overexpression of $\alpha 2$ maintains cardiomyocyte Ca handling and cardiac function in the face of hemodynamic stress, but overexpression of $\alpha 1$ does not (Correll *et al.*, 2014). Hence the principal function of $\alpha 2$ appears to be to control Ca handling by NCX, leaving $\alpha 1$ to maintain bulk intracellular Na.

Subunit-specific localization is not confined to the catalytic α subunit in cardiac muscle. Immunoprecipitation and immunofluorescent experiments have recently indicated that the α_2 subunit predominantly located in t-tubules is accompanied only by the β_2 subunit in human cardiac muscle (Habeck *et al.*, 2016). The t-tubule specific $\alpha_2\beta_2$ pump has a higher sodium affinity than the 'bulk' sarcolemmal $\alpha_1\beta_1$ (9.8mM vs 16mM), a considerably lower potassium affinity (7.4mM vs 1.5mM), and lower turnover rate. At physiological extracellular potassium concentrations its contribution to sodium handling is therefore somewhat small, but the strong voltage dependence of α_2 containing pumps (Crambert *et al.*, 2000, Horisberger and Kharoubi-Hess, 2002) may allow the t-tubular $\alpha_2\beta_2$ pumps to contribute particularly during the cardiac action potential, or at high heart rates if potassium accumulates in cardiac t-tubules as it does in skeletal muscle during prolonged activity (DiFranco *et al.*, 2015).

3.2.2 Relationship between Na pump and mitochondria

As well as directly influencing contractile function via its impact on Ca handling, the Na pump indirectly influences mitochondrial function and therefore ATP supply in cardiac muscle. Mitochondria take up calcium via a uniporter, and extrude it using a Na/Ca exchanger. As calcium rises in the cytoplasm so does mitochondrial matrix calcium and this activates Krebs Cycle dehydrogenases to increase reduction of NAD to NADH, which causes elevated ATP production (Liu and O'Rourke, 2008, Kohlhaas *et al.*, 2010). Mitochondrial ATP production is therefore accelerated during periods of intensive contractile activity. Fast calcium transients in cardiac mitochondria match those in the myocyte cytosol (Maack *et al.*, 2006), enabling mitochondrial Ca to track cytosolic Ca, matching ATP supply to demand. The presence of a Na/Ca exchanger in the inner mitochondrial membrane links cytosolic Na concentration (ie Na pump

function) to mitochondrial Ca (Maack *et al.*, 2006): a rise in intracellular Na activates Ca extrusion from mitochondria via the exchanger in the inner mitochondrial membrane. The impact of reduced mitochondrial calcium is reduced Krebs Cycle flux, which prevents ATP supply meeting demand, leaving the heart metabolically compromised. In myocytes from failing hearts, which have increased intracellular sodium, blockade of the mitochondrial Na/Ca exchanger restores mitochondrial function by enhancing mitochondrial calcium accumulation (Liu and O'Rourke, 2008). Hence as the principal controller of intracellular Na, the pump influences the supply of ATP by cardiac mitochondria, and thus influences all ATP-dependent processes in cardiac muscle.

3.3.3 Pump misregulation in disease

A reduction in the sarcolemmal Na gradient in cardiac myocytes as a result of reduced Na pump function has been observed in both cardiac hypertrophy (Pogwizd *et al.*, 2003, Verdonck *et al.*, 2003a, Verdonck *et al.*, 2003b, Boguslavskyi *et al.*, 2014) and failure (Swift *et al.*, 2008). Many aspects of excitation-contraction coupling are evidently altered in these pathologies. Not all are caused by misregulation of the Na pump, but elevation of intracellular Na does directly contribute to the negative force-frequency relationship, slowed relaxation, arrhythmias, and impaired mitochondrial energetics (Pieske and Houser, 2003, Liu and O'Rourke, 2008, Liu and O'Rourke, 2009) which are hallmarks of cardiac hypertrophy and failure. This raises the interesting possibility that strategies that increase myocyte sodium efflux by restoring or activating Na pump function will restore the Na gradient, and correct many of the functional impairments associated with hypertrophy and heart failure. To date, the aberrant palmitoylation of pump subunits has not been implicated in cardiac pathologies, but this is likely to change, as the field is in its infancy and there is much more work to be done.

Furthermore, aberrant palmitoylation is known to cause or contribute to a wide range of pathologies in the cardiovascular system and beyond: from endothelial barrier dysfunction (Beard *et al.*, 2016) and ischemia-reperfusion injury (Hilgemann *et al.*, 2013, Lin *et al.*, 2013), to cancer (Oo *et al.*, 2014, Tian *et al.*, 2015, Yeste-Velasco *et al.*, 2015), intellectual disability, schizophrenia, Huntington's disease (Young *et al.*, 2012) and diet-induced impairment of synaptic plasticity (Spinelli *et al.*, 2017). We expect that a mechanistic understanding of Na pump regulation by palmitoylation in ventricular muscle will offer the opportunity to manipulate this regulatory process to enhance pump activity for the treatment of diseases such as heart failure.

4. Pump Regulation by the Membrane Bilayer

4.1 Microdomain Localization

In cardiac muscle essentially all active pump is found in caveolae (Liu and Askari, 2006), which are small flask-like invaginations of the cell membrane around 50-100 nm in diameter. Caveolae are a specialized form of lipid raft, enriched in cholesterol and sphingolipids (Brown and London, 2000), characterized by the presence of the oligomeric scaffolding proteins caveolin (caveolin 1 and 3 in cardiac muscle) and cavins. The presence of the Na pump in cardiac caveolae is thought to be facilitated by a caveolin binding motif (CBM (Couet *et al.*, 1997)) in the α subunit transmembrane domain 1, which is required for the interaction between caveolin 1 and the α subunit (Cai *et al.*, 2008). There is unequivocal support from multiple laboratories that the pump directly interacts with caveolin oligomers (Liu *et al.*, 2003, Yosef *et al.*, 2016). However, we find that the presence of PLM modifies the interaction between the pump and the muscle-specific caveolin isoform caveolin 3, with reduced physical interaction between the pump and caveolin 3 in PLM KO hearts, and enhanced interaction between

the two following induction of PLM expression in engineered cell lines (Wypijewski *et al.*, 2015). PLM interacts with the pump α subunit at a considerable distance from the proposed CBM, suggesting that there may be more than one mechanism by which the pump is directed to caveolae (for example palmitoylation of one or more subunits, discussed below). Localization of the cardiac pump to caveolae is necessary to achieve colocalization with the signaling complexes that regulate it: PKA (Rybin *et al.*, 2000, Head *et al.*, 2005), PKC (Rybin *et al.*, 1999), NADPH oxidase (White *et al.*, 2009), and the palmitoyl transferase DHHC5 (Howie *et al.*, 2014). In addition, the phospholipid composition of caveolae (in particular the local enrichment of lipids capable of regulating the pump, such as cholesterol (Brown and London, 2000)) is functionally important for the pump.

4.2 Pump-Phospholipid Interactions

Palmitoylation is the only reversible lipid post-translational modification, and it modifies the relationship between integral membrane proteins and the phospholipid bilayer in which they reside and with which they physically and functionally interact. Palmitoylation of NCX1, for example, sensitizes it to depletion of PIP2 (Reilly *et al.*, 2015, Fuller *et al.*, 2016), while more generally palmitoylation of integral membrane proteins regulates their affinity for lipid rafts (Levental *et al.*, 2010a, Levental *et al.*, 2010b). Like all ion transporters the Na pump interacts with and is influenced by lipids. Recent pump crystal structures have identified several specific lipid-protein interactions that account for the regulatory effects of particular phospholipids on pump activity (Shinoda *et al.*, 2009, Kanai *et al.*, 2013). In order to understand how palmitoylation of pump subunits may modify pump activity it is necessary to first consider how specific lipid-protein interactions regulate the pump.

Palmitoyl carnitine and lysophosphatidylcholine potently inhibit the Na pump (Abe *et al.*, 1984, Pitts and Okhuysen, 1984), whereas long chain fatty acyl CoA derivatives (Kakar *et al.*, 1987) and monoacylglycerols (Askari *et al.*, 1991) stimulate it at physiological concentrations. These stimulatory effects are thought to be due to specific binding of the acyl-CoA to an intracellular domain of the pump, rather than any biophysical effects on the lipid bilayer, or the relationship between the pump and this bilayer (Kakar *et al.*, 1987). While the general physical properties of any lipid bilayer (for example curvature, thickness, elasticity) will inevitably influence the activity of proteins residing within it, specific docking of certain lipids to binding sites on a particular protein offer the opportunity for specific regulation of its activity.

Transmembrane proteins are surrounded by a lipid annulus which can either interact with the surface of the transmembrane domains or exchange with the 'bulk' lipids in the bilayer. Specifically bound, non-annular lipids dock inside the perimeter of this annulus, between transmembrane helices or in binding pockets, in long-lived, high affinity interactions. In the case of ion pumps, channels and exchangers these interactions can regulate activity by stabilizing or destabilizing particular conformations adopted during the reaction cycle. In the case of the Na pump, three principal specific lipid binding sites have been identified in crystal structures, accommodating several phospholipid and cholesterol molecules (Shinoda *et al.*, 2009, Kanai *et al.*, 2013), and accounting for long-established functional effects of these lipids on pump activity.

Na pump activity is regulated by the acidic phospholipid phosphatidylserine (PS) (Wheeler and Whittam, 1970), neutral phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Haviv *et al.*, 2013), sphingomyelin (Habeck *et al.*, 2015) and cholesterol (Cornelius *et al.*, 2003). The specific binding of PS and cholesterol to a site in the α subunit protects the pump from thermal inactivation

without directly modifying its activity (Haviv *et al.*, 2007). PC or PE increase pump activity by specifically binding to a second site (Haviv *et al.*, 2013), also in the transmembrane region. PC or sphingomyelin and cholesterol occupy a third site which causes pump inhibition (Habeck *et al.*, 2015). The PS binding site (referred to as 'site A', Figure 1A (Cornelius *et al.*, 2015)) includes transmembrane domains 8-10 of the α subunit and the FXYD protein transmembrane domain, and accommodates multiple lipids on both extracellular and cytoplasmic sides of the leaflet. The PC/PE binding site ('site B', Figure 1A) lies on the other side of the FXYD protein in a cleft between α subunit transmembrane domains 2, 4, 6 and 9 and the FXYD protein transmembrane domain, on the cytoplasmic side of the bilayer. Notably, the head group of the lipid that occupies site B is positioned very close to the intracellular region of the FXYD protein (Figure 1). The specific interaction of lipids at both sites has recently been elegantly demonstrated by native mass spectrometry of intact α subunit purified from a yeast expression system (Habeck *et al.*, 2017).

The diversity of lipids in biological membranes is not required for either bilayer formation or membrane barrier function, but does offer the potential to regulate the activity of the proteins that reside within them. The existence of specific regulatory binding sites on the Na pump raises the intriguing possibility that acute physiological regulation of pump activity and / or protein turnover can be mediated by the lipid bilayer in which the pump resides. As the only reversible lipid modification, palmitoylation therefore offers the opportunity to dynamically change the relationship between an integral membrane protein and its lipid environment, hence dynamically regulating protein function. The recent finding that changes to the properties of the sarcolemmal membrane can profoundly impact on Na pump activity adds another perspective to the regulatory milieu for the cardiac pump. In murine cardiac myocytes

Ca transients greatly increase Na pump currents at physiological intracellular Na concentrations, via a mechanism that is not fully characterized, but appears to involve changes to the physical properties of the bulk surface membrane (Lu *et al.*, 2016). Given the well-established ability of palmitoylation to modify the relationship between a membrane protein and its lipid environment, this highlights yet another potential regulatory avenue for the pump.

5. Pump Regulation by Post-translational Modifications

5.1 Phosphorylation

5.1.1 Na pump regulation by phosphorylation - conflicting views

Post-translational regulation of the cardiac Na pump by signaling pathways has been extensively studied. Despite this, however, there is remarkably little consensus about either the functional effects or the finer details of the molecular control processes, in particular of kinase-linked regulatory pathways. Indeed, these regulatory pathways and their reported impacts on pump activity vary considerably between different laboratories and model systems. A brief summary of the functional effects of PLM phosphorylation by PKA and PKC on the cardiac Na pump follows, but it is important to appreciate that several investigators have reported that kinases have essentially no effect on pump activity in the heart (Ishizuka and Berlin, 1993, Main *et al.*, 1997, Fine *et al.*, 2013, Lu *et al.*, 2016), while others report that the same pathways described below to activate the pump cause its inhibition (White *et al.*, 2009, White *et al.*, 2010). A detailed discussion of kinase regulation of the pump is beyond the scope of this review. Clearly, the tools and approaches used to date cannot be regarded as satisfactory since they

have generated such diverse and conflicting experimental observations.

Ultimately the relatively mild phenotype of the PLM KO mouse suggests kinase-mediated Na pump regulation via PLM phosphorylation ‘fine-tunes’ rather than drastically modulates pump activity (Bell *et al.*, 2008), implying that the pathways responsible for more profound changes in pump activity remain to be identified. Nevertheless, the PLM KO is more prone to catecholamine-induced arrhythmias (Despa *et al.*, 2008), and a transgenic mouse expressing unphosphorylatable PLM exhibits elevated intracellular Na accompanied by reduced Na pump activity (Pavlovic *et al.*, 2013b, Boguslavskyi *et al.*, 2014). Both observations are consistent with a role for PLM phosphorylation in pump regulation.

5.1.2 PLM phosphorylation

PLM was first identified as an abundant sarcolemmal phosphoprotein in 1985 (Presti *et al.*, 1985a), and was quickly recognised to be the principal sarcolemmal substrate for PKA and PKC in cardiac muscle (Presti *et al.*, 1985a, Presti *et al.*, 1985b). The identification of the FXYP family of pump regulators (Sweadner and Rael, 2000), of which PLM is a member, led to our current understanding that PLM associates with the Na pump in the heart (Crambert *et al.*, 2002, Fuller *et al.*, 2004, Bossuyt *et al.*, 2005, Bossuyt *et al.*, 2006, Bossuyt *et al.*, 2009) and modifies its transport properties.

PLM is phosphorylated at serine 63, serine 68 and serine / threonine 69 by PKC, and at serine 68 by PKA (Walaas *et al.*, 1994, Fuller *et al.*, 2009). Experiments using PLM knockout models clearly demonstrate that the functional effect of PKA and PKC on cardiac Na pump activity requires the presence of PLM (Despa *et al.*, 2005, Han *et al.*, 2006). The precise details of these regulatory events are beyond the scope of this review, but are covered in detail elsewhere (Fuller *et al.*, 2013, Pavlovic *et al.*, 2013a).

Briefly, unphosphorylated PLM inhibits the cardiac Na pump, and this inhibitory effect is relieved or masked following PLM phosphorylation. PLM phosphorylation at serine 68 by PKA increases the sodium affinity of both $\alpha 1$ and $\alpha 2$ containing pump isoforms, while additional phosphorylation of other PLM sites by PKC increases V_{\max} of $\alpha 2$ containing pumps only (Bibert *et al.*, 2008, Bossuyt *et al.*, 2009).

5.2 Palmitoylation

5.2.1 Functional Effects of Palmitoylation on the Na pump

5.2.1.1 FXYP Protein Palmitoylation. Although all Na pump subunits are palmitoylated, only the functional effect of palmitoylation of the FXYP protein has been well studied. In the heart, a biologically meaningful fraction of PLM is palmitoylated, and in intact cells with physiological concentrations of intracellular Na and extracellular K PLM palmitoylation leads to Na pump inhibition, while unpalmitoylated PLM does not exert an inhibitory effect on the pump (Tulloch *et al.*, 2011, Howie *et al.*, 2014). There are two palmitoylation sites just after the PLM transmembrane domain at cysteines in position 40 and 42. Although both are palmitoylated, PEGylation assays (which utilize hydroxylamine-dependent exchange of palmitate for a 5-10kDa PEG molecule to reveal palmitoylation as a band shift on SDS PAGE) indicate PLM is predominantly single palmitoylated in ventricular muscle (Howie *et al.*, 2014). The principal palmitoylation site is cysteine 40: mutation of this site to alanine drastically reduces PLM palmitoylation and the inhibitory effect of PLM on the pump while mutation of cysteine 42 is largely without effect (Howie *et al.*, 2014). Although the functional effects of PLM palmitoylation are relatively modest (~20% inhibition of pump activity), the steep reliance of NCX activity on the transmembrane Na gradient means that even small changes in intracellular Na will

influence Ca handling and hence contractility. Indeed, intracellular Na exerts a greater influence on peak systolic Ca than the activity of any of the cardiac Ca transporters (Hilgemann, 2004), so the change in Na pump activity caused by palmitoylation of PLM C40 by DHHC5 is likely to be functionally significant in the heart.

The localization of the cardiac Na pump to caveolae means that its immediate environment is rich in sphingolipids and cholesterol. All FXYD family members influence the binding of phospholipids to the pump, and PLM in particular stabilizes the interaction between PS and the pump α subunit (site A (Mishra *et al.*, 2011)), albeit with the principal effect being on pump stability rather than its activity. The fact that FXYD proteins in general and PLM in particular can modify the relationship between the pump α subunit and specifically bound phospholipids leads us to speculate that the inhibitory effect of PLM palmitoylation on the pump is mediated by changes in pump-phospholipid interactions. The close proximity of the PC/PE head group in the α subunit lipid stimulatory site (site B) to PLM cysteine 40, coupled with the need for the acyl chain of the conjugated palmitate to be accommodated in the lipid bilayer suggests that palmitoylation of PLM at cysteine 40 alters the ability of PC/PE to occupy site B and / or stimulate ion transport by the α subunit (Figure 1B). In support of this concept, experiments in which recombinant FXYD proteins were reconstituted with the Na pump *in vitro* suggest that in different lipid environments recombinant PLM is capable of both activating or inhibiting the pump. Reconstitution of PLM with recombinant $\alpha 1\beta 1$ in stearoyl-oleoyl-phosphatidylserine (SOPS) and cholesterol (Cirri *et al.*, 2011) or dioleoyl phosphatidylserine (DOPS) (Lifshitz *et al.*, 2006) increases the pump's Na affinity compared to that in the absence of PLM. However when the same proteins are reconstituted in liposomes consisting of a mix of phospholipids with a broad distribution of fatty acid chains (comparable to a cell membrane), PLM reduces

pump Na affinity (Cirri *et al.*, 2013). A component of PLM-induced pump activation could be explained by the stabilizing effect of PLM on the pump enzyme complex (Lifshitz *et al.*, 2007, Mishra *et al.*, 2011). Ultimately however, since recombinant (unpalmitoylated, unphosphorylated) PLM can act as both a pump activator or inhibitor depending on the lipids used for reconstitution, it is conceivable that palmitoylated PLM exerts its effect on pump activity in a physiological setting by changing the relationship between the pump complex and its specifically bound lipids.

Palmitoylation also controls the turnover rate of PLM: unpalmitoylatable PLM is degraded more rapidly than the wild type protein. This occurs independently of an effect on the degradation rate of the α subunit (Tulloch *et al.*, 2011), suggesting that PLM palmitoylation does not influence steady state turnover and therefore abundance of the pump.

5.2.1.2 α and β Subunit Palmitoylation. Experimental evidence (largely from proteomic screens) suggests that several isoforms of the α subunit (Yang *et al.*, 2010, Dowal *et al.*, 2011, Forrester *et al.*, 2011, Martin *et al.*, 2011, Wilson *et al.*, 2011, Serwa *et al.*, 2015, Fang *et al.*, 2016, Hernandez *et al.*, 2016, Pinner *et al.*, 2016) and all β subunits (Kang *et al.*, 2008, Martin *et al.*, 2011) of the pump are also palmitoylated in various tissues and cell lines. The functional effects of palmitoylation of either subunit on pump activity and/or localization, as well as relevance to the cardiac enzyme remains to be determined. The sole non-extracellular cysteine in the β 1 subunit of the pump (cysteine 46) resides within the transmembrane region, but it must become solvent-exposed even if only transiently in order for the side chain to encounter a palmitoylating enzyme. Meanwhile several palmitoylation sites have been identified in the α 1 subunit. The most interesting of these is C374 adjacent to the catalytic aspartate (D376), which becomes

transiently phosphorylated during the pump's reaction cycle. A cysteine in position -2 to this aspartate is a common feature of many P-type ATPases. Although the structural imposition of a membrane anchor so close to the catalytic core of the enzyme would be expected to influence pump activity, to date no functional effect has been ascribed to palmitoylation of the α subunit of the pump. However direct functional regulation of other P-type ATPases by palmitoylation of their α subunits has recently been reported in *Aspergillus nidulans* (Zhang *et al.*, 2016): the DHHC-PAT AkrA controls intracellular Ca signaling by regulating the activity of two P-type ATPases. Hence palmitoylation of the catalytic subunit may prove to be a universal regulatory feature for this class of enzymes.

5.2.2 Palmitoylating Enzymes

Our understanding of the forward reaction of palmitoylation, catalyzed by Asp-His-His-Cys motif containing palmitoyl acyl transferase enzymes (DHHC-PATs), is in its infancy compared to other post-translational modifications, but will be aided by the recently-described crystal structure of DHHC20 (Rana *et al.*, 2018). These transmembrane enzymes, which are found in all compartments of the secretory pathway, are first palmitoylated within their catalytic site (the cysteine of the DHHC motif) following transfer of palmitate from palmitoyl CoA, before the palmitate is transferred to substrate proteins (Jennings and Linder, 2012). DHHC-PATs appear to fall into two functional groups. Some form a stable complex with their substrate, with palmitate transfer dependent on this interaction and hence highly selective. The binding of other DHHC-PATs to their substrates is barely detectable, yet palmitoylation of these substrates is in some cases more efficient (Lemonidis *et al.*, 2014). Included in the latter group are the Golgi-localized enzymes DHHC3 and DHHC7. The ankyrin-repeat

containing DHHC-PATs DHHC13 and DHHC17 are in the former group (Lemonidis *et al.*, 2015), as is the enzyme responsible for palmitoylation of PLM, DHHC5.

Although most DHHC-PATs share a common transmembrane structure, with 4 transmembrane domains and a highly conserved active site in a cysteine-rich intracellular loop, the intracellular amino and carboxyl termini show little homology between family members. DHHC5 and the closely related enzyme DHHC8 have very long (~500 residue) carboxyl tails, which are predicted to be highly disordered (Howie *et al.*, 2014). We used a high throughput co-immunoprecipitation approach to identify DHHC-PATs that form a stable complex with PLM. DHHC-PATs 4, 5, 6 and 7 were found to interact with PLM to a much greater extent than any other DHHC isoforms. DHHCs 2, 4 and 5 are the most abundantly expressed in the heart at the level of mRNA, and DHHC5 predominantly localizes to cardiac caveolae, where it interacts with PLM (Howie *et al.*, 2014). Overexpression of DHHC5 in cultured cells enhanced palmitoylation of PLM, while silencing it largely abolished PLM palmitoylation. Hence while PLM may be palmitoylated in the secretory pathway by DHHC-PATs that it encounters and forms stable complexes with (predominantly DHHCs 4, 6 and 7), when resident at the plasma membrane the principal determinant of its palmitoylation status is the presence of DHHC5. Not only does this identify DHHC5 as a regulator of the cardiac Na pump via PLM, the co-localization of PLM with its acyl transferase in cardiac caveolae (along with the rapid phosphorylation-induced change in PLM palmitoylation: see section 5.3) also strongly suggests that palmitoylation of PLM is dynamic rather than static, as has previously been reported for the related protein FXYD5 (Martin *et al.*, 2012).

Truncation analysis identified a region between N218 and T334 of the DHHC5 extended C tail that is required for DHHC5 to recruit PLM. Deletion of this region,

which is predicted to be highly disordered, abolished the physical interaction between DHHC5 and PLM, and prevented the palmitoylation of PLM by DHHC5. Hence the formation of a stable complex between DHHC5 and PLM is required and precedes palmitoylation of PLM by DHHC5.

Disorder predictions indicate that the majority of human DHHC-PATs consist of a core ordered cytosolic cysteine rich domain with relatively disordered intracellular N and C termini. This is consistent with the concept that regions outside the cysteine-rich domain are responsible for substrate recruitment to DHHCs (Greaves *et al.*, 2009, Huang *et al.*, 2009, Nadolski and Linder, 2009). In general disordered domain interactions therefore likely underlie substrate recognition by (and hence substrate specificity of) some DHHCs. Specifically this occurs between the intracellular carboxyl tails of PLM and DHHC5.

So what regulates DHHC5 palmitoylation of PLM? For now, this remains unknown. However, both PLM and DHHC5 are known to undergo multiple post-translational modifications in the intracellular regions that interact with each other. It is therefore tempting to speculate that regulation of palmitoylation is at the level of the interaction between enzyme and substrate, rather than enzymatic activity of DHHC5 itself. No post-translational modifications have yet been identified that regulate palmitate turnover in a DHHC-PAT active site. A model in which palmitoylation by DHHC5 is regulated by substrate recruitment to the enzyme is consistent with that which has been established for DHHC5 in the central nervous system, where δ -catenin palmitoylation is regulated by interaction and co-localization with DHHC5 (Brigidi *et al.*, 2014, Brigidi *et al.*, 2015), albeit only following the internalization of DHHC5 via clathrin coated vesicles mediated by adaptor protein binding to its C tail. Notably the available data regarding DHHC5 in the heart suggest that unlike in the brain, it resides

in the same membrane compartment as its substrates, for example in caveolar microdomains with PLM. We speculate that one important difference between the role of DHHC5 in the brain and the heart is the timescale of responses. Synaptic plasticity tends to both develop and persist for longer periods of time than acute changes in cardiac output / function.

5.2.3 Depalmitoylating Enzymes

Only a small number of protein thioesterases have been identified to date, one of which (PPT1) is lysosomal (Linder and Deschenes, 2007, Tomatis *et al.*, 2010). The cytosolic serine hydrolase APT1 (LYPLA1) has been proposed to depalmitoylate H-ras and G protein α subunits (Duncan and Gilman, 1998, Duncan and Gilman, 2002). However, pharmacological inhibition of this enzyme does not cause global changes in the abundance of palmitoylated proteins (Dekker *et al.*, 2010) prompting consideration of other serine hydrolases as contributing to palmitoylation turnover and dynamics (Martin *et al.*, 2012). Indeed the recent discovery that $\alpha\beta$ -hydrolase 17 depalmitoylates PSD-95 and N-ras suggests that the breadth of depalmitoylating enzymes is much greater than had been initially thought (Lin and Conibear, 2015, Yokoi *et al.*, 2016). Although palmitoylation of PLM likely turns over relatively quickly (since it is rapidly palmitoylated upon phosphorylation – see below), the identity of the depalmitoylating enzyme remains un-reported.

5.2.4 Indirect effects of palmitoylation on the pump

5.2.4.1 Massive Endocytosis. The role of DHHC5 in cellular and cardiac biology has received attention recently with the finding that a novel form of endocytosis, massive endocytosis (MEND), in which up to 70% of the cell surface membrane is internalized, is controlled by DHHC5 (Fine *et al.*, 2011, Hilgemann and Fine, 2011, Lariccia *et al.*,

2011, Hilgemann *et al.*, 2013, Lin *et al.*, 2013). Calcium overload leading to mitochondrial stress causes transient openings of the mitochondrial permeability transition pore (MPTP), releasing of coenzyme A into the cytoplasm where it is acylated to form a substrate for DHHC5 to palmitoylate surface membrane proteins (Hilgemann *et al.*, 2013). The clustering of acylated proteins in lipid ordered domains leads to MEND by as-yet unidentified mechanisms in multiple cells types (Reilly *et al.*, 2015, Fuller *et al.*, 2016).

Importantly, MEND occurs during reperfusion of anoxic cardiac muscle (Lin *et al.*, 2013), is accelerated in the presence of PLM (Hilgemann *et al.*, 2013), and is inhibited by interventions classically reported to reduce MPTP opening and protect against reperfusion injury, such as adenosine (Liu *et al.*, 1991) and cyclosporin A (Baines *et al.*, 2005). DHHC5 knockout hearts in which MEND is significantly reduced show enhanced functional recovery following anoxia-reperfusion (Lin *et al.*, 2013), strongly implicating DHHC5 and the MEND pathway in cardiac reperfusion injury. Hence palmitoylation of cardiac substrates by DHHC5 controls cellular processes that underlie a significant worldwide health burden. Of particular relevance to the cardiac Na pump and cardiac ion transport in general is the finding that this type of endocytosis preferentially internalizes pumps and ion transporters, probably because these proteins have bulky intracellular and small extracellular regions that favor their clustering in invaginated curved membrane domains (Reilly *et al.*, 2015, Fuller *et al.*, 2016). Indeed, palmitoylated proteins prefer to reside in such highly curved membranes (Larsen *et al.*, 2015).

These observations highlight another means by which activity of DHHC5 may be regulated – by the availability of its substrate palmitoyl CoA. However, it is important to distinguish between ‘pathological’ activation of DHHC5, which remodels

the cell membrane via MEND, and the ‘physiological’ role of DHHC5, in regulating ion transport by the pump. In the absence of MEND-inducing stressors and global remodeling of the cellular palmitome, palmitoylation of PLM by DHHC5 modifies pump activity, but not its abundance at the cell surface (Howie *et al.*, 2014).

3.2.4.2 Palmitoylated PLM Oligomers. PLM is not only found associated with the Na pump in cardiac muscle, it also interacts with itself. The pool of oligomeric PLM that does not interact with the pump can be distinguished by its almost stoichiometric phosphorylation at serine 63 by PKC (Wypijewski *et al.*, 2013). Dephosphorylation of oligomeric PLM is without effect on pump activity. Indeed, despite its presence in caveolar membranes with the Na pump, the PLM oligomer may not be functionally linked to the pump, or capable of exchanging with pump-associated PLM. PP2A resides in close proximity to pump-associated PLM thanks to its interaction with the pump α subunit (Kimura *et al.*, 2011). This population of PP2A maintains the pump-associated pool of PLM dephosphorylated at serine 63, as PP2A is capable of dephosphorylating PLM serine 63 but not serine 68 or threonine 69 (El-Armouche *et al.*, 2011).

As well as displaying a different phosphorylation signature, oligomeric PLM is also notably more heavily palmitoylated than the pump-associated pool (Wypijewski *et al.*, 2013). PLM oligomerises in many cell types and membrane environments (Moorman *et al.*, 1995, Song *et al.*, 2011), and FRET measurements suggest that the PLM oligomer is a tetramer (Song *et al.*, 2011). A PLM tetramer can be modeled based on the 4-helix parallel leucine zipper (PDB code 1C94 (Mittl *et al.*, 2000)) between four monomeric PLM transmembrane domains (PDB code 2JO1 (Teriete *et al.*, 2007), Figure 2). PLM leucines 16, 27, 30, 33 and 34, and isoleucines 23 and 26 contribute to the zipper, while the side chains of phenylalanine 28 (which interacts with the pump α subunit (Khafaga *et al.*, 2012)) and cysteine 42 face the bilayer on the outside of the of

the zipper, with cysteine 40 orientated inside. Palmitoylation at cysteine 40 would therefore not be accommodated in the PLM tetramer, while palmitoylation at cysteine 42 would. This suggests that the PLM palmitoylation site responsible for regulating the pump (cysteine 40) can also regulate the formation of the PLM tetramer, although a physiological role for this tetramer remains to be identified.

5.3 Relationship Between PLM Phosphorylation And Palmitoylation

Although unpalmitoylatable PLM is phosphorylated normally by PKA and PKC (Tulloch *et al.*, 2011), there is a relationship between PLM palmitoylation and its phosphorylation by PKA. Paradoxically, phosphorylation of PLM at S68 by PKA increases PLM palmitoylation (Tulloch *et al.*, 2011). Hence one post-translational modification of PLM that activates the Na pump, promotes a second that inhibits it. There are many examples of phosphorylation and palmitoylation interacting with each other on the same target protein (Tian *et al.*, 2008, Salaun *et al.*, 2010, Gauthier-Kemper *et al.*, 2014, Moritz *et al.*, 2015), but palmitoylation usually inhibits phosphorylation (and vice versa) because one modification attracts proteins to membranes while the other repels them. PLM phosphorylation close to its C terminus either increases the mobility of its intracellular C tail to allow DHHC5 to access the juxtamembrane palmitoylation sites, or increases the interaction between PLM and DHHC5 to promote PLM palmitoylation. The consequences for the cardiac Na pump of increased PLM palmitoylation following its phosphorylation by PKA remain unclear because in cardiac muscle we cannot distinguish whether C40 (the Na pump regulatory cysteine) or C42 in PLM is palmitoylated following PKA activation. If, for example, PKA-induced palmitoylation of PLM merely increases the half life of PLM without influencing Na pump activity, the resulting increase in cell surface expression of PLM would simply increase the number of Na pumps associated with PLM, possibly increasing the ability

of the pump to respond to a subsequent kinase challenge. Alternatively, if PLM palmitoylation influences the formation of the PLM oligomer, PKA-induced palmitoylation may modify the ability of PLM to oligomerise but not alter pump activity.

6. Conclusions & Perspectives

There is much still to learn about Na pump regulation by palmitoylation. In particular, we don't know how PLM is depalmitoylated in cardiac muscle. Nor do we have a full picture of how DHHC5 recruitment and palmitoylation of PLM is regulated; although PLM phosphorylation and palmitoyl-CoA availability clearly have a role to play, other mechanisms and pathways are also likely to contribute. The apparently paradoxical enhancement of PLM palmitoylation by phosphorylation (a post-translational modification thought to activate the pump) requires further investigation. The disordered C tail of DHHC5 is rich in predicted sites of a diverse range of post-translational modifications. Whether recruitment to / recognition of PLM by DHHC5 is regulated by post-translational modification to the enzyme's C-tail remains to be determined, but seems likely. Indeed, whether DHHC5 interacts directly with PLM or through another pump subunit, has yet to be definitively shown. In addition, although the palmitoylation sites and functional effect of palmitoylation of PLM are relatively well understood, all subunits of the cardiac Na pump are palmitoylated and the functional consequences of palmitoylation of the pump's α and β subunits remain unknown.

In conclusion, a picture emerges of palmitoylation as a mechanism that tunes the activity of the cardiac Na pump via the associated FXYD protein PLM. This relationship is likely to have general significance as all FXYD proteins possess

juxtamembrane cysteines (Tulloch *et al.*, 2011), which may also be palmitoylated. As our understanding of the relationship between the Na pump and its lipid bilayer becomes ever clearer, so will the molecular details of how PLM palmitoylation inhibits the pump, and how we can intervene for therapeutic gain. The therapeutic potential of targeting palmitoylation of individual proteins has recently been demonstrated for the melanocortin-1 receptor (MC1R): inhibiting depalmitoylation of MC1R variants associated with melanoma prevents melanomagenesis (Chen *et al.*, 2017). Ultimately if we can exploit our developing understanding of the cellular events leading to Na pump inhibition by palmitoylation, we hope to find that the relationship between DHHC5 and PLM is similarly ripe for exploitation to manipulate Na pump activity to ameliorate cardiac pathologies.

Declaration of Interest

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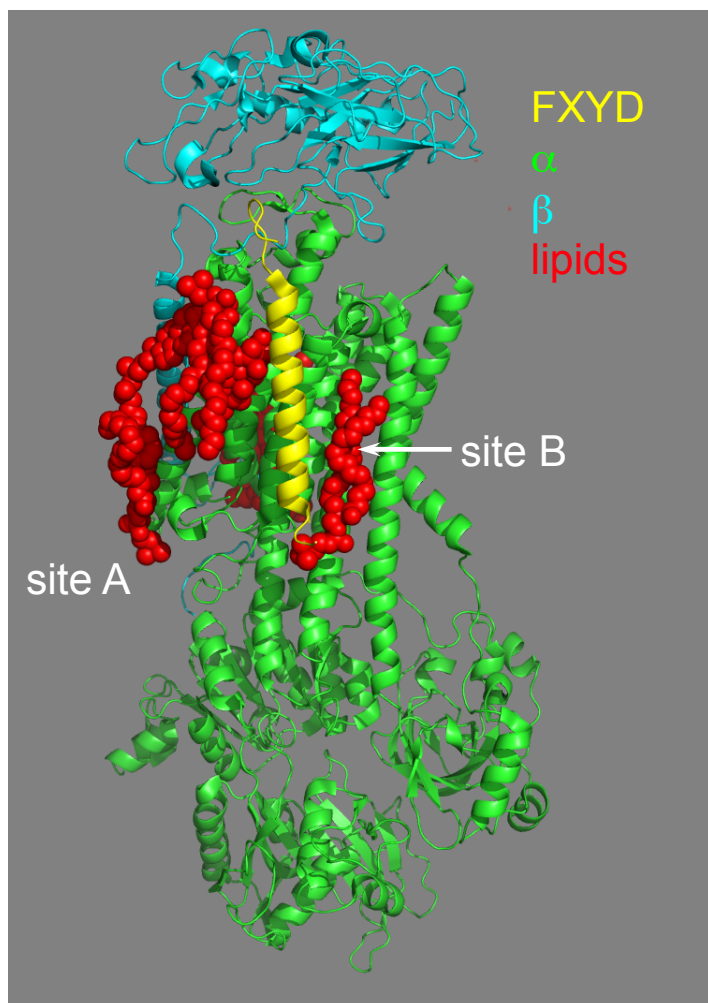
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Figure 1. Relationship between the Na pump and specifically-bound regulatory lipids. **A:** Crystal structure of the Na pump purified from the pig kidney, showing α (green), β (cyan) and γ (FXYP2, yellow) subunits, based on PDB 3WGV (Kanai *et al.*, 2013). Specifically bound lipids are shown occupying sites A (stabilizing) and B (stimulating). The third lipid binding site is obscured by the α subunit. **B:** The stimulatory lipid occupying site B lies in very close proximity to the FXYP protein (PLM in cardiac muscle). The intracellular C tail of the FXYP protein is not resolved, and structural information ceases at an arginine equivalent to R39 in PLM. We speculate that palmitoylation of PLM C40 influences the ability of lipid to occupy site B and / or stimulate the α subunit.

Figure 2. Model of the PLM tetramer based on the 4-helix parallel leucine zipper (PDB code 1C94 (Mittl *et al.*, 2000)). Each PLM monomer is presented in a different color. **A:** The transmembrane helical domains form a coiled coil which is fastened by the leucine zipper. Key residues are shown in sphere representation. F28, which interacts with the Na pump α subunit, and palmitoylatable C42 both protrude from the outside of the 4-helix coiled coil, while regulatory C40 points inwards. **B:** The transmembrane region with one PLM subunit cut away to show the leucine zipper. Leucine and isoleucine residues point inwards to grasp one another. **C:** View down the center of the 4-helix parallel leucine zipper from the extracellular space showing leucine/isoleucine residues packing tightly together.

A**B**