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Review article

Novel regulation of cardiac Na pump *via* phospholemman ☆

Davor Pavlovic [a,*], William Fuller [b], Michael J. Shattock [a]

[a] Cardiovascular Division, King's College London, The Rayne Institute, St Thomas' Hospital, London, UK
[b] Division of Cardiovascular & Diabetes Medicine, Medical Research Institute, College of Medicine Dentistry & Nursing, University of Dundee, UK

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ABSTRACT

As the only quantitatively significant Na efflux pathway from cardiac cells, the Na/K ATPase (Na pump) is the primary regulator of intracellular Na. The transmembrane Na gradient it establishes is essential for normal electrical excitability, numerous coupled-transport processes and, as the driving force for Na/Ca exchange, thus setting cardiac Ca load and contractility. As Na influx varies with electrical excitation, heart rate and pathology, the dynamic regulation of Na efflux is essential. It is now widely recognized that phospholemman, a 72 amino acid accessory protein which forms part of the Na pump complex, is the key nexus linking cellular signaling to pump regulation. Phospholemman is the target of a variety of post-translational modifications (including phosphorylation, palmitoylation and glutathionation) and these can dynamically alter the activity of the Na pump. This review summarizes our current understanding of the multiple regulatory. This article is part of a Special Issue entitled "Na^+ Regulation in Cardiac Myocytes".

Contents

1. Introduction

Since the discovery of Na/K ATPase (Na pump) in 1957 [1] a major research effort has been focused on investigating the structural and regulatory properties of this ubiquitous P-type ATP-driven cation transporter. The Na pump uses the free energy of hydrolysis of ATP to exchange three intracellular Na ions for two extracellular K ions, thus setting the electrochemical gradient for both Na and K across the cell membrane. The Na pump is therefore vital for maintaining

Abbreviations: NO, nitric oxide; PKC, protein kinase C; PKA, protein kinase A; PLM, phospholemman; PLB, phospholamban; ET-1, endothelin 1; ET_A, endothelin A receptor; NOS, nitric oxide synthase; AR, adrenergic receptor; PP-1, phosphatase-1; NCX, Na/Ca exchanger; I-1, inhibitor-1.

* Corresponding author at: Cardiac Physiology, The Rayne Institute, St Thomas' Hospital, London SE1 7EH, UK. Tel.: +44 2071888351; fax: +44 2071880970.
E-mail address: davor.pavlovic@kcl.ac.uk (D. Pavlovic).

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the resting potential and Na and K gradients in almost every eukaryotic cell. These gradients ensure basic cellular homeostasis such as regulation of cell volume, essential ionic and amino acid transport processes. In excitable cells Na pump activity restores the Na and K gradients following depolarization and in the kidney its activity provides the driving force for Na reabsorption essential to control extracellular volume and blood pressure. Among the many Na-dependent transmembrane transport processes in muscle cells, the activity of the Na pump drives Na/Ca exchanger (NCX) and thus regulates contractility. This review will focus on the regulation of the cardiac Na pump at the protein and enzyme level and specifically, regulation by its accessory protein phospholemman.

1.1. Structure of the Na pump

Our understanding of the structure–function relationship has greatly expanded with the discovery [2] and refinement [3–5] of Na pump crystal structures. The Na pump is a multi-subunit enzyme composed of 3 subunits, α, β and a member of a FXYD family [6]. The α subunit, with 10 transmembrane segments, contains the binding sites for Na, K, ATP and cardiotonic steroids such as ouabain and digoxin. The minimum functional unit is made up of α and β macromolecular complex and there are four isoforms of the α subunit (α1, α2, α3 and α4) and three of the β subunit (β1, β2 and β3) [7,8]. The catalytic function of Na pump for transport of Na and K ions relies on the α subunit, whereas the association with β subunit is required for the complex to traffic through the secretory pathway to the plasma membrane [9,10]. Each of the α and β isoforms is encoded by their own gene and can potentially form 12 different Na pump isozymes with distinct transport and pharmacological properties [11]. In the heart α1 isoform is the dominant, ubiquitous isoform, whereas α2 and α3 are present in smaller amounts and their expression differs between species. In rodents, α1 and α2 are the two main isoforms [12], whereas dogs and macaques express α1 and α3 [13]. In human hearts all three α isoforms are detected [13] with estimates ranging from stoichiometric distribution [14] to α1 being dominant (62%) over α2 (15%), and α3 (23%) [15], although it is unclear to what extent this represents a 'pure' myocyte population and how much of it is a "contamination" from non-myocytes. In addition to the α and β subunit, in most tissues it is now well recognized that a third FXYD subunit (originally designated γ in the kidney) forms part of the pump complex. Seven members of the FXYD family have been identified in mammalian tissues and these tissue specific accessory proteins provide further diversity to the Na pump function and structure [6]. While the Na pump can function in the absence of FXYD subunit both *in vivo* and *in vitro* [16,17], it is not clear whether the FXYD subunit is ever absent from the α/β complex under physiological conditions, in a cell that has not been genetically modified. Thus, most researchers agree that in the heart a functional Na pump complex is made up of α1 or α2 subunits [18,19] in association with β1 and FXYD-1, although α3 [20,21] and β3 [22] subunits have been detected. Whereas, FXYD1 (commonly referred to as phospholemman) is regularly detected in both cardiac homogenates and myocytes [23] FXYD5 has only been detected in homogenates [24], therefore, it is unclear whether it originates from myocyte or non-myocyte population.

1.2. Role of Na pump in the heart

In the heart, intracellular Na is regulated by balance of Na influx and efflux mechanisms. While there are many influx pathways, the Na pump provides the only significant Na efflux pathway and is therefore vital for the maintenance of normal electrical activity and the Na gradient. This Na gradient drives the activity of many co-transporters and exchangers including the NCX. Thus by controlling steady-state intracellular sodium, the Na pump regulates the concentration of intracellular Ca *via* NCX, which in turn determines the content of sarcoplasmic reticulum (SR). An increase in Na would limit 'forward mode' Na/Ca exchange (Na in, Ca out) and possibly even favoring more Ca influx and less Ca efflux, resulting in a larger Ca transient and therefore increased contractility [25]. This is the accepted mechanism of action for the inotropic effect of cardiotonic steroids (Na pump inhibitors) used to increase cardiac output in patients with congestive heart failure ever since their effects were first described by William Withering in 1785. In addition to its transport function, evidence is accumulating that Na pump also plays a signaling role [26], whereby, cardiotonic steroid binding to the extracellular region of the pump α-subunit activates early-response genes associated with cell growth (see review by Li and Xie [27]). Whether this is independent of inhibition of its transport function and the accompanying changes in intracellular Na and Ca are equivocal [28,29]. While the role of cardiotonic steroids in normal physiology is yet to be understood, data is accumulating in support of their role in disease (see review by Lingrel [30]).

We have previously shown that α1 isoform provides around 88% of the total Na pump current and is relatively evenly distributed within the cells, whereas, α2 is 5 times more concentrated in the t-tubules compared to sarcolemma [31]. Although t-tubule membranes represent only 30% of total surface area they generate approximately 41% of the total Na pump current, approximately 70% of α2 and 37% of α1 pump current. Nevertheless, α1 pump current still dominates in t-tubules with α1:α2 density ratio of 4:1 [31], although 1:1 ratios have also been reported [32]. In light of differential distribution of the α1 and α2 in the cardiac cell, it has been suggested that α1 and α2 isoforms have different physiological roles within the cardiac myocytes. Recent experiments using SWAP mice, where ouabain sensitivities of α1 and α2 subunits have been reversed, suggest that indeed, α2 has a more prominent role (*vs.* α1) in modulating cardiac myocyte SR Ca release [33]. As both α1 and α2 are physically and functionally associated with NCX in cardiac myocytes [34,35] it is tempting to speculate that α2 isoform controls the local Na and thus Ca levels (*via* NCX) in sarcolemma/sarcoplasmic reticulum microdomains whereas α1 pumps maintain a global pool of Na throughout the cell. However, considering that at least 50% of the t-tubular Na pump current is generated by the α1 pumps in mouse ventricular myocytes [31,32], it seems more likely that while α1 subunits are dominant in controlling global Na, both α1 and α2 control Na microenvironment at the sarcolemma/sarcoplasmic reticulum junction and hence SR Ca release (see Fig. 1).

1.3. Na pump in the diseased heart

While it is well established that Na pump is vital for maintenance of trans-membrane Na gradient in a healthy heart, there is accumulating evidence that disruption of this gradient may play a role in the development of ischemia/reperfusion [36,37], hypertrophy and heart failure [38–42]. During ischemia, increases in intracellular Na concentration are attributed to a combination of Na influx *via* late activating Na channels [43] and increased activity of Na/H exchanger [44] as well as a decreased efflux through Na pump [45]. Whereas, the relative contributions of Na influx *versus* efflux have not yet been determined it is clear that the rise in intracellular Na during ischemia and its failure to recover completely on reperfusion are likely to strongly influence the electrical and contractile dysfunction in the ischaemic/reperfused myocardium [37,46]. Similarly, increased intracellular Na concentration is well established during heart failure, however, it still not clear what causes this Na accumulation. Increased Na influx *via* Na channels was reported in rabbit [47], dog [48,49] and human failing hearts [49], whereas, Na/H exchanger activation was implicated by Baartscheer et al. using a rabbit heart failure model [50,51]. There is also considerable literature suggesting compromised Na extrusion (*via* the Na pump) in heart failure. Our data from human heart failure samples show significantly lower phospholemman Ser68 phosphorylation but no change in total phospholemman, or α-1

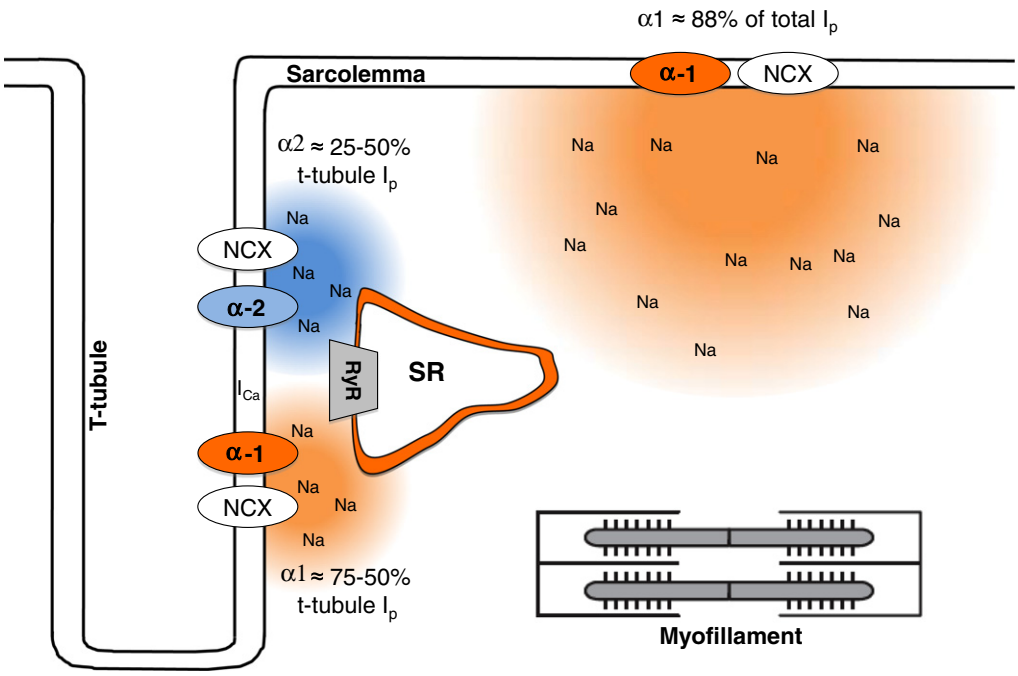

Fig. 1. Cartoon depiction of relative distributions of Na pump α1 and α2 subunits in a cardiac myocyte. Na pump resides and regulates intracellular Na and Ca (*via* NCX) in both sarcolemma and t-tubules in cardiac myocytes. α is evenly distributed within the cells and is the dominant isoform providing around 88% of the total Na pump current (I_p). α2 is 5 times more concentrated in the t-tubules compared to sarcolemma and provides only around 12–24% of the total I_p but possibly up to 50% of the I_p in the t-tubules. Thus, α1 subunits are dominant in controlling global Na, whereas, both α1 and α2 control Na microenvironment at the sarcolemma/sarcoplasmic reticulum junction.

subunit expression, compared to healthy donor hearts [23]. This is in agreement with some studies conducted on human, canine and rat heart failure models [21,41,52] but certainly not all. Bossuyt et al. have reported a reduction in total phospholemman and α-1 subunit expression in rabbit heart failure model but no change in α-1 subunit expression in human heart failure samples [20]. Schwinger and colleagues have reported a decrease in α- and α-3 subunits along with decreased Na pump activity in human heart failure [53]. Nevertheless, despite differences in pump and phospholemman expression observed, a reduction in Na pump activity and an increase in intracellular Na were reported in almost all of the above studies. In the short term, this might limit systolic dysfunction (by increasing sarcoplasmic reticulum Ca) and thus be beneficial to the failing heart [40,54]. However, a chronic increase in intracellular Na and Ca is associated with maladaptive cardiac hypertrophy and arrhythmogenesis [55–57].

2. FXYD proteins — Tissue specific regulators of the Na pump

Considering that Na pump has to fulfill not only its ionic "housekeeping" duties but also plays a pivotal role in many other specialized biological processes it is not surprising that its regulation at the protein and enzymatic levels is complex. Some of the well characterized Na pump regulators include, intracellular sodium, extracellular potassium, ATP, membrane potential and cardiotonic steroids. In addition, recent experimental evidence has revealed a novel regulatory mechanism that involves interaction of the Na pump with small-membrane proteins of the FXYD family. A little over 10 years ago, Sweadner and Rael defined the so-called FXYD protein family, based on the signature sequence containing the Phe-X-Tyr-Asp (FXYD) motif, two conserved glycines and one serine residue [6]. The mammalian FXYD family contains 7 members that include FXYD1 (phospholemman) [58], FXYD2 (γ-subunit) [59], FXYD3 (mammary tumor marker, Mat-8) [60], FXYD4 (corticosteroid hormone-induced factor, CHIF) [61], FXYD5 (related to ion channel RIC or dysadherin) [62], FXYD6 (phosphohippolin) [63] and FXYD7 [64]. They are all type I membrane proteins with a single transmembrane domain, an extracellular NH_2 terminus and a cytoplasmic COOH terminus. Except for FXYD2 and FXYD7, all are predicted to contain a cleavable NH_2-terminal signal peptide. All of the FXYD members contain 61–95 amino acids (with the exception of FXYD5, which has 178 amino acids due to a terminal extension at its amino terminus) and transmembrane domains of FXYDs 1, 2, 3, and 4 were all shown to adopt an α-helical conformation [65,66]. For many years after their discovery, the function of FXYD proteins was unknown. Several FXYD proteins were shown to induce ion-specific conductances when overexpressed in Xenopus oocytes [60,61,67] but it is still controversial whether this has any physiological significance. It wasn't until the discovery that FXYD2 associates and modulates renal Na pump activity in 1997 [68–70] that the research community focused on investigating their effects on the Na pump. Although it is not clear whether FXYD proteins might have other functions, at least five of the FXYD proteins associate with and regulate Na pump activity in a tissue specific manner (see review by Geering [71]). Thus there is a general consensus that FXYD proteins are accessory proteins to the α/β pump complex, allowing for tissue-specific regulation of the pump, tailored to the needs of the environment the pump is required to regulate.

3. Na pump regulation by phospholemman

Phospholemman is a small single membrane-spanning protein (72 amino acids), mainly expressed in the heart, skeletal and smooth muscle but also in other tissues such as brain liver and kidneys [58,72–74]. It was initially suggested that phospholemman forms taurine-selective channels in lipid bilayers [75] and that it therefore might be involved in cell volume regulation. While some structural studies support the idea of phospholemman multimers [76,77], myocytes from both wild type (PLM^{WT}) and phospholemman knock out (PLM^{KO}) mice swell equally, indicating that phospholemman is not essential in limiting water accumulation in response to a hypo-osmotic challenge [78]. That said, a pool of pump-free phospholemman multimers has recently been described in ventricular muscle, which may represent a 'storage pool' of phospholemman that does not directly regulate the pump [79]. Classification of phospholemman as a FXYD protein pointed to its

Na pump regulatory role. Unlike the other FXYD proteins it contains multiple, well conserved, phosphorylation sites at its COOH terminus. Indeed, phospholemman is a principal sarcolemmal substrate for PKA at Ser68 and PKC at residues Ser63, Ser68 and Ser/Thr69 [80,81]. NIMA (never in mitosis A) kinase has also been reported to phosphorylate phospholemman at Ser63 although the functional effects of this kinase on the Na pump have never been identified.

Physical interaction between phospholemman and the α subunit of the Na pump in the heart has been demonstrated using co-immunoprecipitation [20,76,82–84], crosslinking [85] and most recently, FRET [82,86]. The interaction between the phospholemman and α/β complex was also observed in crystal structures from shark rectal gland [3] and pig kidney Na pump [2]. However, presumably due to the mobility of the carboxyl terminus of phospholemman, they have provided no structural insight on the pump-phospholemman interactions at the intracellular region of phospholemman. Solution and solid-state NMR spectroscopy studies in micelles and bilayers suggest that the cytosolic tail (helices 3 and 4) of the unphosphorylated phospholemman is tightly associated with the negatively charged phospholipids of the membrane [87,88]. Initial NMR experiments with phospholemman phosphorylated at Ser68 by PKA indicate that phosphorylation increases the dynamics around helix 4 [88], however, whether phosphorylation is accompanied by detachment of helix 4 from the lipid surface is yet to be determined using NMR spectroscopy. Elegant work by Khafaga and colleagues, using FRET, showed that E960 residue on the pump and F28 on phospholemman are critical for phospholemman-mediated effects on both pump function and physical pump-phospholemman interaction [86]. It should be noted that mutation of the E960-F28 residues did not completely abolish physical interaction between the pump and phospholemman, indicating that there are other, as yet undiscovered interaction sites that hold the two together. Nevertheless, there is a general agreement between the FRET, cross-linking and co-immunoprecipitation studies that phosphorylation alters the association between the pump and phospholemman by moving the cytosolic arm away from the pump but not by promoting their dissociation (see Fig. 2).

Functional effects of phospholemman on the cardiac Na pump have been confirmed by several independent laboratories using a range of approaches and experimental models. Unphosphorylated phospholemman inhibits the cardiac Na pump whereas phosphorylation by either PKA or PKC stimulates it. However, there is some disagreement regarding the exact nature of this modulatory effect on the pump. Unphosphorylated phospholemman was shown to inhibit Na pump activity either *via* a decrease in apparent Na affinity [82,83,89–92], decrease in V_{max} [16,80,93,94] or both [90,95]. It is likely that some of these discrepancies are due to differences in methodology employed between different labs. For example, in experiments where changes in V_{max} are reported [16], pipette Na concentration was 50 mM. While at this concentration any changes in Na pump activity can be mainly attributable to V_{max}, it is not possible to rule out an effect on the apparent Na affinity. It is also likely that high basal phospholemman phosphorylation, which in a freshly isolated cardiac myocyte is a mixture of Ser63 (circa 57%), Ser68 (circa 33%), both Ser63/Ser68 (circa 28%) and unphosphorylated phospholemman (circa 38%) is another potential source of error [80]. Considering that basal phosphorylation is a result of a combination of the activities of both PKC (dictated by resting Ca load) and PKA (dependent on the adrenergic state of the cell), and there is evidence that PKA stimulates the pump *via* increase in apparent Na affinity and PKC *via* increase in V_{max} [90,95], these can clearly provide another layer of complexity to the interpretation of data. Despite disagreements over the V_{max}/K_M effects, which still persist, introduction of PLM^{KO} mice removed doubts over the mechanism of pump modulation. In myocytes isolated from the PLM^{KO} animals, pump current (I_p) was higher than in PLM^{WT} and supplementation of PLM^{WT} with phosphorylated phospholemman peptide increased I_p up to the levels observed in PLM^{KO} myocytes [16]. Furthermore, phosphorylation of phospholemman *via* beta and alpha-adrenoceptor resulted in increased I_p and Na extrusion rates in PLM^{WT} mice but had no effect in PLM^{KO} [89,90]. These studies

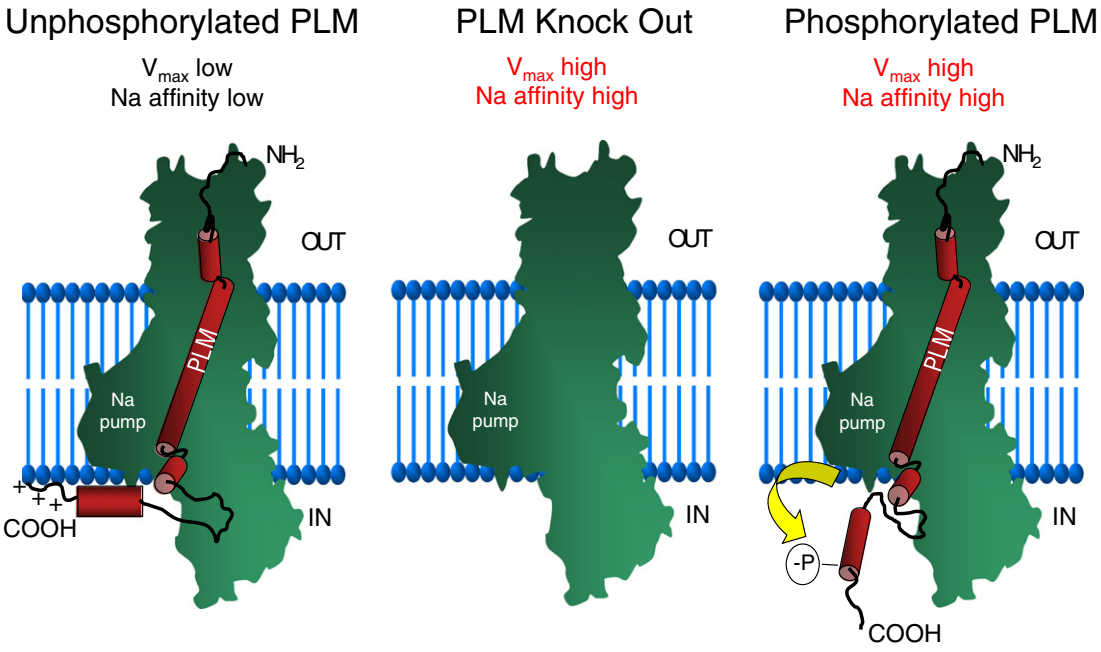

Fig. 2. Hypothetical cartoon depiction of a structure–function relationship between phospholemman (PLM) and Na pump α-subunit. The cytoplasmic tail of unphosphorylated PLM interacts closely with the membrane and α-subunit of Na pump, whereas, phosphorylation alters the association between the pump and PLM by moving the cytosolic arm away from the pump, but not by promoting their dissociation. Phosphorylation or ablation of phospholemman relieves inhibition of the Na pump by increasing its V_{max} and apparent Na affinity (adapted from Shattock [56]). Therefore under stress, phosphorylation of phospholemman allows the heart to reduce its Na and Ca load and thus prevents lethal arrhythmias.

showed that unphosphorylated phospholemman acts as a brake on the Na pump and that phosphorylation removes this brake by changing the orientation of the phospholemman cytosolic carboxyl terminus with respect to the pump α subunit, thereby increasing its activity (see model in Fig. 2).

3.1. PKA signaling and phospholemman

In the heart, β-receptor stimulation increases inotropy and lusitropy by increasing L-type Ca current, increasing movement of Ca back into the SR (*via* phospholamban phosphorylation), increasing the rate of Ca dissociation from the myofilaments (phosphorylation of troponin I) and increasing intracellular Na (*via* increased heart rate). Furthermore, β-receptor stimulation was consistently shown to increase the activity of the cardiac Na pump [16,80,84,93,96–99]. Surprisingly some laboratories also report inhibition [100,101], however, this could be a result of sub-physiological intracellular Ca concentration used in these studies (<150 nM) [102]. It should also be noted that there is a correlation between PKA-mediated Ser936 phosphorylation on the α-1 subunit and Na pump inhibition, however, relevance of this mechanism to cardiac Na pump regulation is questionable as all the studies were performed in non-cardiac tissues (for review see Poulsen et al. [103]). On the contrary, there is strong evidence that PKA-dependent phosphorylation of phospholemman at Ser68 residue mediates β-receptor activation of the cardiac Na pump [16,23,76,80,82,83,89,93]. This conclusion was reinforced by experiments in PLM^{KO} mice in which I_p was reduced in PLM^{WT} compared to PLM^{KO} myocytes, while the addition of the PKA-phosphorylated 19 amino acid peptide corresponding to the carboxyl terminus of phospholemman increased I_p [16]. Conversely, addition of the unphosphorylated phospholemman peptide reduced the I_p in both PLM^{WT} and PLM^{KO} myocytes [16]. Surprisingly, phosphorylated peptide further stimulated Na pump in PLM^{KO}, however, it is possible that this is an artefact of using a phospholemman peptide that does not contain the transmembrane domain. Indeed, Lifshitz et al. have reported that transmembrane domain alone exerts some inhibitory effect on the Na pump [92]. Further experiments by Despa and colleagues unequivocally showed that β agonist isoprenaline stimulated Na pump activity only in PLM^{WT} but not PLM^{KO} myocytes [89], indicating that phospholemman is required for the PKA-mediated stimulation of the Na pump (see Fig. 3).

While the evidence for β-adrenergic stimulation of the Na pump seems solid, an inotropy paradox is apparent. Increased Na pump activity during fight or flight seems counterintuitive as it would lead to reduced intracellular Na (and therefore Ca) and thus to reduced inotropy. So the mechanism (fight or flight) responsible for increasing cardiac output also initiates a mechanism (phospholemman phosphorylation) potentially reducing cardiac output? Despa and colleagues provided the answer in 2008 by measuring the effect of β-AR activation on intracellular Na and Ca in myocytes from PLM^{WT} and PLM^{KO} mice. An increase in stimulation frequency plus β-adrenoceptor activation caused a larger rise in intracellular sodium, greater SR Ca content, and bigger Ca transient in PLM^{KO} compared to PLM^{WT} myocytes. However, greater SR Ca content led to more arrhythmias in isolated myocytes from PLM^{KO} animals [104]. It should be noted that Despa and colleagues found a 20% downregulation of the α subunit in PLM^{KO} myocytes, suggesting higher apparent Na affinity (due to the absence of PLM) and reduced pump expression in PLM^{KO} mice might offset each other so that under physiological conditions, Na pump activities in PLM^{KO} and PLM^{WT} are comparable [89]. The difference occurs after sympathetic stimulation, where the pump is activated in PLM^{WT} but not in PLM^{KO} myocytes thus allowing for PLM^{WT} to decrease their intracellular Na load. Remarkably, we do not observe such adaptational changes in our PLM^{KO} colony and find that I_p is around 30% higher in PLM^{KO} then in PLM^{WT} [16,31], but in PLM^{3SA} mice where phospholemman Ser63, Ser68 and Ser/Thr69 residues were mutated to alanines (and thus these animals cannot upregulate their Na pump activity following β-adrenergic stimulation), we find similar increases in Ca load and propensity to arrhythmias under sympathetic stress, as observed by Despa and colleagues (see Fig. 4). Thus, the physiological role of phospholemman may be to limit the rise in intracellular Na during sympathetic stimulation and thereby prevent Ca overload and triggered arrhythmias in the heart. This is supported by *in vivo* measurements of dP/dt in PLM^{KO} and PLM^{WT} mice [105], Wang and colleagues reported increased +dP/dt both at baseline and at low concentrations of isoprenaline in PLM^{KO} mice [105].

3.2. PKC signaling and phospholemman

PKC to a large extent mimics the effect of PKA activation on the Na pump in the heart. Most studies show that PKC stimulates the pump [80,90,95,106,107], however, inhibition [108–110] was also reported. In the kidney, intracellular Ca has been shown to interfere with the

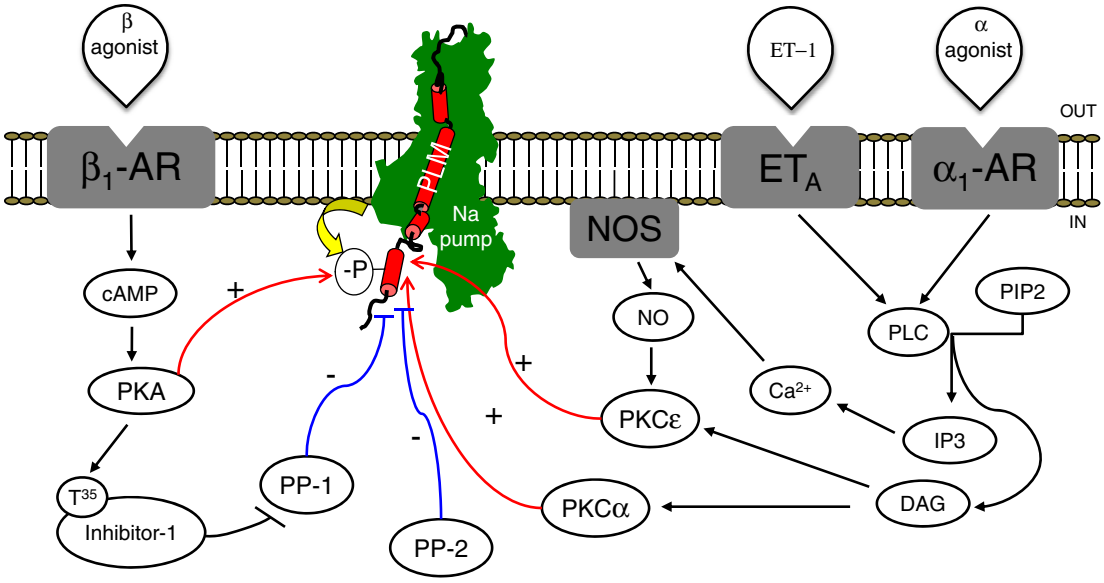

Fig. 3. Signaling pathways regulating Na pump activity *via* phosphorylation of PLM. PLM phosphorylation state is regulated by the kinase activity of PKA and PKC and phosphatase activity of PP-1 and PP2-A. PKA and PKC phosphorylate phospholemman and thus stimulate Na pump (denoted by +) whereas PP-1 and PP2-A remove phosphates from phospholemman and thus inhibit the pump (denoted by −). ET-1, endothelin 1; ET_A, endothelin A receptor; NOS, nitric oxide synthase; AR, adrenergic receptor.

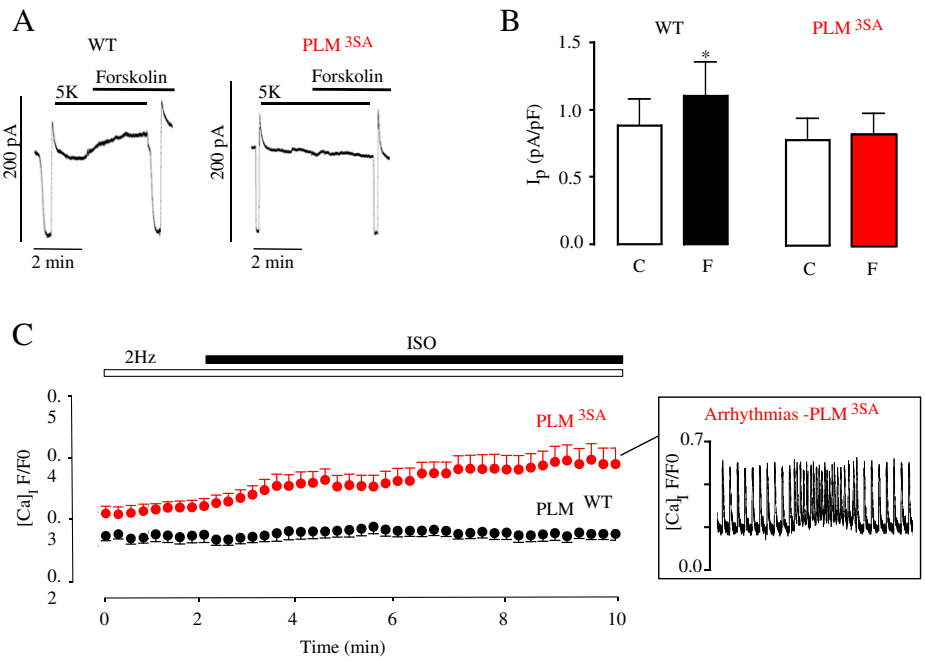

Fig. 4. Field-stimulation of PLM^{3SA} mouse myocytes results in elevation of diastolic Ca^{2+} and arrhythmias. (A) Forskolin increases Na pump current in mouse myocytes isolated from PLM^{WT} but not in mice where phospholemman residues Ser63, Ser68 and Ser69 were mutated to alanines (PLM^{3SA}), as shown in perforated whole-cell patch clamp studies (raw traces shown on the left). 0 mM of K was used to inhibit the Na pump and 5 to activate. (B) Graph of forskolin (denoted as F) induced pump current changes in mouse myocytes and non-treated controls (denoted as C). (C) Time course of changes in diastolic Ca induced by field-stimulation (2 Hz) and 1 μmol/L isoprenaline in ventricular myocytes from PLM^{WT} and PLM^{3SA} mice. Inset: PLM^{3SA} mice show higher susceptibility to arrhythmias. Adapted from Pavlovic et al. [112].

functional effects of PKC on the Na pump [111] and indeed we have shown this to be the case in the heart [112]. It is therefore possible that discrepant effects of PKC on the pump are a function of differences in intracellular Ca used. There are at least three PKC isoforms expressed in the heart α, δ and ε [80], providing further source of complexity. Nevertheless, Na pump activation by PKC was shown to be dependent on PLM [90], although the mechanism for this increase in pump activity is still not clear as changes in V_{max} alone [90,95] or both V_{max} and K_m were reported [82]. Bibert et al. have examined the effects of PKA and PKC activation on the Na pump in Xenopus oocyte expression system and found that while PKA activation (*via* phospholemman) increases apparent Na affinity of both α1 and α2 pump isoforms, PKC had no effect on the apparent Na affinity of either α1 or α2 but increased the maximum turnover rate of the α2 pumps only [95]. Bossuyt et al. study used SWAP mice (where α1 and α2 ouabain sensitivities are swapped), and found that either PKA or PKC activation increased Na affinities (*via* phospholemman) of α1 and α2 isoforms in cardiac myocytes. However, PKC activation increased V_{max} of α2 but not α1 isoform [82], similar to experiments in oocytes. Based on these findings, it was proposed that PKA and PKC have access to different pools of PLM, Ser68 and Ser63, respectively, and thus maintain intracellular Na differently in the vicinity of either α1 or α2 isoforms. Although there is evidence that PKA is functionally linked to the α1 isoform [31,113], data on the exclusive link between PKC and α2 are ambiguous. In mouse ventricular myocytes, activating PKA after PKC induces additional Ser68 phosphorylation and increases pump Na affinity (on top of a PKC-induced increase in V_{max}) whereas, activating PKC after PKA induces Ser63 and additional Ser68 phosphorylation and increases pump V_{max} (on top of a PKA-induced increase in Na affinity) [90]. Similarly, in Xenopus oocytes where rat phospholemman and α1 or α2 isoforms were overexpressed, PKA-induced Ser68 phosphorylation increased the apparent Na affinities of both α1 and α2 isoforms, whereas, PKC phosphorylation (of Ser63 and Ser68 residues) increased the V_{max} of α2 but not α1 isoforms [95]. These data indicate that both α1 and α2 isoforms can "sense" Ser68 phosphorylation (resulting in a change in the apparent Na affinity of the pump), whereas, α2 isoforms can additionally sense Ser63 phosphorylation (resulting in an increased V_{max}). However, it is difficult to explain how α2 isoform, which provides only 12% of the total pump current [31], can account for the 60% increase in pump V_{max} observed when PKC is activated [90]. Furthermore, phosphorylation by PKA or PKC activation induced similar reductions in FRET between YFP-labeled phospholemman and CFP-labeled α1 or α2 subunits, indicating that there is no difference in the physical association between the phospholemman and α subunits following phosphorylation. However, submaximal concentrations of PKA agonists had a smaller effect on the FRET between phospholemman and α2 than α1, suggesting subtle differences in association and regulation of Ser68 residue and the two Na pump isoforms. Thus, despite recent advances in our understanding of PKC mediated phospholemman regulation, the physiological role of PKC-induced Na pump stimulation has not been established although our work on the nitric oxide (NO) mediated pump regulation may provide some insights (NO signaling and phospholemman section), see Fig. 3 for mechanism.

3.3. NO signaling and phospholemman

Raising intracellular Ca^{2+} either artificially or *via* field-stimulation in ventricular myocytes activates constitutively expressed nitric oxide synthase (NOS), generating NO in submicromolar concentrations [114,115]. Confusingly, NO has been reported to mediate both inhibition [116–121] and stimulation of the Na pump [122–127]. An elegant hypothesis explaining these apparent discrepancies was proposed, stating that NO stimulates Na pump only in tissues expressing phospholemman [128]. However, as is often the case, "*Another beautiful hypothesis destroyed by an ugly fact*", some of the tissues where NO was reported to inhibit the pump were subsequently found to express phospholemman [129]. Nevertheless we have shown that in field-stimulated cardiac myocytes, NO activates the Na pump *via* PKCε-induced phosphorylation of PLM at Ser-63 and Ser-68 residues, in a Ca-dependent manner (see Fig. 3 for mechanism). Furthermore, in patch-clamped myocytes, NO increased apparent Na affinity of the pump in PLM^{WT} but not PLM^{KO} animals, again confirming the

requirement of phospholemman for the pump stimulation. The resulting Na pump stimulation was found to play an important role in protecting the heart against Na^+ and Ca^{2+} overload (*via* NCX) and resultant arrhythmias in both field-stimulated cardiac myocytes and hearts in the absence of sympathetic stimulation. We propose that in a beating heart, intracellular Na and thus Ca are basally controlled through phospholemman-induced Na pump regulation (*via* NO) whereas fight or flight-induced activation of the pump activity is a "reserve", only used following sympathetic stimulation in order to deal with increased Na and Ca influx (see PKA signaling and phospholemman section). The effects of PKA and PKC activation are additive, both in terms of phosphorylation of phospholemman and functional effects on the Na pump [90] and indeed β-receptor-induced phosphorylation of Ser68 phospholemman residue was shown to be enhanced by NO-mediated PKCε activation [112].

3.4. Phosphatases and phospholemman

While phosphatases have often been considered to be the poorer cousins of the kinases, and have tended to elicit less excitement amongst the scientific community, they are equally important and as well regulated as kinases in living systems. Despite suggestions that phosphatases are involved in regulation of phospholemman as early as 1999 [130], relatively little research has been conducted to date on the pathways leading to phospholemman dephosphorylation. In the heart more than 90% of total phosphatase activity is contributed by the types 1 and 2A, PP-1 and PP-2A [131]. In particular PP-1 has been implicated in the regulation of cardiac β-agonist responses and as a negative regulator of cardiac contractility [132–134]. It is the major phosphatase dephosphorylating the SERCA-pump regulator phospholamban (PLB) [131], and thereby negatively affects Ca transients [134]. PP-1 activity in turn is tightly regulated by several regulatory and a few inhibitory subunits with the latter including inhibitor-1 (I-1) and inhibitor-2. The cytosolic I-1 [134–136] is activated by cAMP/PKA-dependent phosphorylation at Thr-35 and then potently prevents substrate-dephosphorylation by PP-1. We have recently shown that phospholemman phosphorylation at Ser-68 and cardiac Na pump activity is negatively regulated by PP-1 and that this regulatory mechanism is counteracted by PKA-dependent I-1, under resting conditions [23] (see Fig. 3). Using okadaic acid as a crude phosphatase inhibitor we found that IC_{50} for inhibition of Ser-63 dephosphorylation is much lower (127 nM) than for Ser-68 (525 nM) indicating that PP-2A might be responsible for Ser-63 dephosphorylation. Interestingly, the EC_{50} for the Thr-69 site was high, 2.7 μM, indicating that even if Thr-69 dephosphorylation may be mediated by PP1, this is unlikely to occur under physiological conditions. Furthermore, phospholemman phosphorylation was diminished in failing human hearts selectively at the PKA-dependent Ser-68 residue, which is consistent with impaired β-AR signaling, I-1 deactivation and potentially hyper-activated PP-1, as previously reported in these heart failure samples [137,138].

Recently, we have shown that presence of the PP-2A in the pump complex in cardiac muscle maintains pump-associated phospholemman unphosphorylated at Ser-63 [79]. Therefore, it is possible that differential exposure of pools of phospholemman to different phosphatases can drive different phosphorylation patterns for pump-associated and pump-free phospholemman although more work is required to understand what effect these phenomena might have in determining trafficking and functional roles of phospholemman within the cardiac cell.

3.5. Palmitoylation of phospholemman

Phospholemman has two cysteines at residues 40 and 42 (which lie in the intracellular region of phospholemman just beyond the transmembrane domain) that are completely conserved across species. Cysteine 42 has recently been reported to be glutathionylated during oxidative regulation of the cardiac sodium pump [139] and we have recently reported that both Cys40 and Cys42 are palmitoylated [22]. Palmitoylation of phospholemman increases the half-life of protein and importantly decreases Na pump activity, probably through a modification of the local environment surrounding the entrance to the sodium binding sites in the α subunit [22]. Surprisingly, phosphorylation of phospholemman at serine 68 by PKA in rat ventricular myocytes or transiently transfected HEK cells increased its palmitoylation [22]. Considering that Ser68 phosphorylation is predicted to increase Na pump activity and palmitoylation to decrease it, it is difficult to predict the physiological significance of these two seemingly opposing regulatory mechanisms. It has been suggested that individual palmitoylation sites on phospholemman may have opposing effects on pump activity through their reorienting effects on phospholemman helix 3 (where these sites are located), thus providing further complexity to this regulatory mechanism [140]. Clearly more research is needed to understand the role of palmitoylation in phospholemman mediated pump regulation, however, considering that one or both cysteines are found in analogous positions throughout the FXYD family [141], and all are predicted to be palmitoylated [22], FXYD protein palmitoylation may be a universal means to regulate the pump.

3.6. Oxidant stress and phospholemman

Oxidant stress alters protein structure and function through the modification of the redox status of regulatory protein sulfhydryl groups. As early as 1993, Shattock and Matsuura have found that Na pump current was reduced by photoactivated rose-bengal (a singlet oxygen and superoxide generator) in voltage-clamped rabbit ventricular myocytes [142]. Furthermore, depletion of cellular glutathione or intracellular application of thiol-modifying reagents reduced pump activity [143], providing more evidence in support of a possible functional link between pump activity and its protein sulfhydryl status. While there is agreement that oxidant stress inhibits Na pump function, mechanisms driving this process are unclear. Glutathionylation of the cardiac pump has been shown to occur on α [144] and β [110,145] subunits, and both are reported to negatively regulate Na pump function. The cardiac β1 subunit was shown to be glutathionylated (at Cys46 residue) either by application of oxidants (peroxynitrite or hydrogen peroxide) [145], following activation of PKCε-dependent NADPH oxidase [110] or surprisingly even *via* activation of PKA signaling cascades [100]. Consequent inhibition of the cardiac Na pump is mediated by a decrease in maximal turnover rate of the pump. Phospholemman is reported to reverse this β1-glutathionylation-mediated pump inhibition by acting as a "decoy" for oxidant stress, being itself glutathionylated at Cys42 residue [139]. It is very difficult to interpret this data in the context of well-reported stimulatory effects of PKA [16,80,84,93,96–99] and PKC [80,90,95,106,107] on the Na pump activity. Adding further confusion to an already complicated picture is evidence that oxidant stress can activate PKA in ventricular myocytes [146,147] leading to substantial phosphorylation of phospholemman at S68. Reconciling the opposing effects of phosphorylation, palmitoylation and glutathionylation on the pump activity is not straightforward. It is possible that differences in methodology, basal phosphorylation state of the isolated myocytes and intracellular Ca levels can account for some, but certainly not all the divergent results. With increasing realization of the importance of Na pump regulation in both normal physiology and disease, it is imperative that these differences are addressed if we are to progress to designing clinically effective therapeutic strategies.

4. Phospholemman as a therapeutic target

It is clear from the literature that Na overload can contribute to contractile and electrical dysfunction in ischemia/reperfusion [36,37], hypertrophy and heart failure [38–42]. Reduction of Na influx was already shown to reduce infarct size [148], arrhythmias [149] and ischemic injury [43], so as the Na efflux pathway is also compromised in

hypertrophy [38,41,42] and heart failure [23,150], it seems reasonable to suggest that this may also be considered as a therapeutic target. In fact, as pointed out by Rasmussen and Figtree in their analysis of drug therapies targeting the neurohormonal abnormalities in heart failure patients, there is a remarkable correlation between the ability of the treatment to stimulate the Na pump and its clinical outcome [151]. Thus, increases in Na pump activity, through the modulation of phospholemman, may provide an important therapeutic target in cardiovascular disease. There is an argument that in heart failure and ischemia where the heart is already energetically compromised, increasing the activity of energy demanding Na pump could further burden the cell and thus make matters worse. However, there is accumulating evidence that high intracellular Na contributes to the impaired mitochondrial energetics [152,153] through diminished mitochondrial Ca uptake [154] required to stimulate ATP synthesis. Therefore therapeutically, it might be favorable to reduce Na overload and allow the cell to start producing more ATP.

5. Conclusions

Owing to the importance of Na pump as a major efflux pathway for Na and its role in driving a plethora of other transporters in almost every single cell in our body, it is perhaps not surprising to find a complex network of regulators and "fine tuners" controlling its activity. Multiple regulatory pathways converge on phospholemman, some of them seemingly cancelling each other, and some of them acting synergistically (see Fig. 5 for a summary of functional effects of various phospholemman modifications). Phospholemman-phosphorylation mediated effects on the pump have now been reproduced by many independent groups and the role of phospholemman Ser68 residue in protection against β-receptor-mediated Na/Ca overload and arrhythmias is well characterized. However, accumulating data on cysteine modifications *via* palmitoylation and glutathionylation is compelling and detailed studies addressing the contribution of each of the three regulatory pathways are urgently needed. Perhaps an important factor being overlooked in our quest to understand the role of phospholemman-mediated Na pump regulation is the old adage in the real estate thesaurus "location, location, location". We are becoming increasingly aware that cardiac cells are not "empty bags" waiting to be filled but complex structures with strictly localized protein networks precisely regulating their immediate environment. Perhaps if we develop the tools to study and modify individual populations of molecular networks in different areas of t-tubules, caveolae or sarcolemma, we will discover that phosphorylation, palmitoylation and glutathionylation are not mutually exclusive.

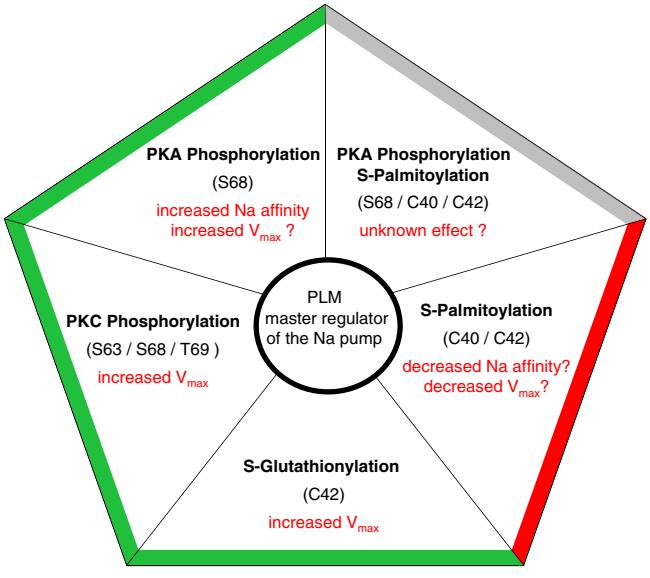

Fig. 5. Summary of the many post-translational modifications of PLM and their functional effects on the Na pump activity. The functional effect of each modification on pump activity (compared to unmodified PLM) is indicated by color shown on the outer face of the panels, green for Na pump activation, red for inhibition, or grey where effect is not known. Adapted from Fuller et al. [140].

6. Disclosure

None.

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