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Cardiac hypertrophy in mice expressing unphosphorylatable phospholemman

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Aims	Elevation of intracellular Na in the failing myocardium contributes to contractile dysfunction, the negative force–frequency relationship, and arrhythmias. Although phospholemman (PLM) is recognized to form the link between signalling pathways and Na/K pump activity, the possibility that defects in its regulation contribute to elevation of intracellular Na has not been investigated. Our aim was to test the hypothesis that the prevention of PLM phosphorylation in a PLM ^{3SA} knock-in mouse (in which PLM has been rendered unphosphorylatable) will exacerbate cardiac hypertrophy and cellular Na overload. Testing this hypothesis should determine whether changes in PLM phosphorylation are simply bystander effects or are causally involved in disease progression.
Methods and results	In wild-type (WT) mice, aortic constriction resulted in hypophosphorylation of PLM with no change in Na/K pump expression. This under-phosphorylation of PLM occurred at 3 days post-banding and was associated with a progressive decline in Na/K pump current and elevation of [Na] _i . Echocardiography, morphometry, and pressure-volume (PV) catheterization confirmed remodelling, dilation, and contractile dysfunction, respectively. In PLM ^{3SA} mice, expression of Na/K ATPase was increased and PLM decreased such that net Na/K pump current under quiescent conditions was unchanged (cf. WT myocytes); [Na ⁺] _i was increased and forward-mode Na/Ca exchanger was reduced in paced PLM ^{3SA} myocytes. Cardiac hypertrophy and Na/K pump inhibition were significantly exacerbated in banded PLM ^{3SA} mice compared with banded WT.
Conclusions	Decreased phosphorylation of PLM reduces Na/K pump activity and exacerbates Na overload, contractile dysfunction, and adverse remodelling following aortic constriction in mice. This suggests a novel therapeutic target for the treatment of heart failure.
Keywords	Na/K ATPase • Phospholemman • Hypertrophy • Intracellular sodium

1. Introduction

In the heart, the Na/K ATPase is crucial for the maintenance of the normal transmembrane Na gradient. A reduction of this gradient has been implicated in a variety of pathologies including ischaemia/reperfusion,^{1,2} hypertrophy, and heart failure (HF).^{3–5} In hypertrophy and HF, many aspects of excitation–contraction (E–C) coupling are clearly altered; however, the elevation in intracellular Na may contribute to (i) the negative force–frequency relationship (FFR), (ii) slowed relaxation, (iii) arrhythmias, and (iv) impaired mitochondrial energetics.^{6,7} While a component of the elevation of intracellular Na may reflect an increase in Na influx,⁸ there is a large body of evidence showing that Na/K pump function may also be compromised.^{3–5,9} Specifically, in

cardiac hypertrophy, we (and others—see Pogwizd *et al.*³ and Verdonck *et al.*⁵ for reviews) have shown that Na/K pump function is reduced.

It is now well established that FXYP proteins are tissue-specific regulators of Na/K ATPase activity. In the heart, FXYP1, or phospholemman (PLM), has now been extensively characterized and has been shown to be an essential mediator of Na/K pump activation in response to cellular signalling cascades [specifically protein kinase A (PKA) and protein kinase C (PKC)]. Unphosphorylated PLM exerts a tonic inhibition on the pump which is relieved by PLM phosphorylation of one, or all of three, residues on its cytoplasmic tail [serines (Ser) Ser63, Ser68, or Ser/Thr69]. This phosphorylation is hence important for the dynamic control of intracellular Na during increases in heart rate and prevention of Na overload during 'fight or flight'.^{10–12} In addition to PLM, a range of other ion regulation or E–C

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coupling proteins, such as L-type Ca channels, phospholamban (PLB), the ryanodine receptor (RyR), and troponin I, are all phosphorylated by the activation of PKA, PKC, or NO-dependent pathways. Although acute inotropic activation of these pathways helps increase the Ca transient while maintaining efficient relaxation, chronic activation may lead to Ca overload, diastolic dysfunction, and arrhythmias.^{13,14}

It is now widely accepted that sympathetic activation is an acute and maintained response to cardiac overload and is a critical component of the progression into HF. In the minutes and hours following a myocardial infarction, for example, this immediate sympathetic outflow may be necessary to maintain cardiac output, blood pressure, and peripheral perfusion. However, the sustained long-term activation of the sympathetic nervous system is now recognized as one of the detrimental aspects of the progression towards HF and as such β -blockade is the first-line therapy. The SOLVD studies show that even in asymptomatic patients, plasma catecholamines are elevated.¹⁵ The up-regulation of sympathetic outflow is assumed to be an *immediate* response to myocardial stress and as such it seems reasonable to hypothesize that, in the minutes, hours, and days following acute stress, PKA substrates will be *hyperphosphorylated*. Although it has been reported that the hyperphosphorylation of the sarcoplasmic reticulum (SR) Ca-release channel RyR2 persists, the majority of studies suggest that, after a poorly described initial peak, the phosphorylation of PLB declines to below baseline as the β -adrenoceptor signalling pathway down-regulates.^{16–18}

Despite the importance of PLM in the regulation of intracellular Na, and the considerable evidence suggesting intracellular Na is elevated in hypertrophied and failing myocardium and contributes to diastolic dysfunction, the role of PLM and its phosphorylation in the overloaded and failing heart has not been systematically characterized. Bossuyt *et al.*¹⁹ have reported hyperphosphorylation of PLM in a rabbit model of volume overload-induced dilated failure, whereas El-Armouche *et al.*²⁰ have reported hypophosphorylation of PLM in failing human hearts—attributable to down-regulation of inhibitor-1 and increased protein phosphatase 1 (PP-1) activity. This lack of consensus may reflect different models or different stages of the disease process. It is also evident that simply reporting up- or down-regulation of PLM phosphorylation does not distinguish between an effect that causally influences the progression of the disease and one that is simply a bystander—reporting dynamic disease-induced changes in PKA and PKC signalling pathways.

The primary aim of this study was therefore to test the hypothesis that the specific prevention of PLM phosphorylation in a PLM knock-in mouse (in which PLM has been rendered unphosphorylatable) will exacerbate cardiac hypertrophy and cellular Na overload. Testing this hypothesis should determine whether any changes in PLM phosphorylation are simply bystander effects or are causally involved in the progression of the disease. To do this, we have generated a knock-in mouse (PLM^{35A}) in which the PLM residues phosphorylated by PKC and PKA¹⁰ (Ser63, 68, and 69) have been mutated to alanines. To meet this principle aim, we have initially characterized changes in PLM expression and phosphorylation, and functional indices of hypertrophy, Na pump function and intracellular Na, in genetically normal C57BL/6J mice subjected to suprarenal aortic constriction. We have then established the baseline characteristics of the PLM^{35A} mouse heart and determined response to aortic constriction of the PLM^{35A} mice and their wild-type (WT) littermates.

2. Methods

Full details of the Methods used in this study are given in Supplementary material online.

2.1 Animals and PLM^{35A} mice

All experiments were performed in accordance with the Guidance on the Operation of Animals (Scientific Procedures) Act, 1986 (UK), the Directive of the European Parliament (2010/63/EU), and received approval by the local ethics review board. Male WT mice (C57BL/6J) or male PLM^{35A} knock-in mice, or their WT littermates, were used. PLM^{35A} mice express an unphosphorylatable form of PLM in which Ser63, 68, and 69 have all been mutated to alanines (see Supplementary material online for more detail). This mutated PLM gene is expressed under the control of the endogenous *FXD-1* promoter, resulting in normally regulated levels of PLM globally expressed in tissues that usually express PLM (i.e. heart, smooth muscle, skeletal muscle etc.). Initial hypertrophy studies (in which sham and banded animals were compared) were conducted in C57/Bl6 mice (Harlan UK, Ltd). Heterozygote breeders were used to generate homozygote PLM^{35A} mice and they were compared with WT littermates.

2.2 Hypertrophy model

Myocardial hypertrophy was induced by pressure overload following suprarenal aortic constriction (banding) in 6-week-old C57BL/6J mice (20–22 g), PLM^{35A} knock-in mice, or their WT littermates.²¹ The reproducibility of the aortic constriction, and hence pressure overload, was assessed by post-mortem measurement of the residual luminal area (RLA) and constrictions falling outside predefined limits were excluded (see Supplementary material online).

For all surgical procedures and echocardiography, mice were anaesthetized by inhalation of isoflurane/O₂ mixture (2/98%). Adequacy of anaesthesia was controlled by monitoring corneal reflex and respiration (rate, depth, and pattern of breathing). Acute postoperative analgesia was a single buprenorphine (Vetergesic 0.3 mg/mL solution) injected i.p. at dose 20 μ g/kg. Euthanasia [after invasive pressure–volume (PV) measurements] was performed by overdose of isoflurane. Death was confirmed by monitoring cardiac activity and respiration. Heart excision (for myocyte isolation or *in vitro* assessment of cardiac function) was performed after terminal anaesthesia by i.p. injection of pentobarbital (Pentoject 200 mg/mL solution) at dose 300 mg/kg and heparin (150 U).

2.3 Assessment of cardiac function

Cardiac function was measured *in vivo* in anaesthetized mice 5 and 9 weeks after surgery in sham and banded animals. Cardiac dimensions and contractility were measured using 2D echocardiography (Visualsonics Vevo 770 ultrasound system with a 30 MHz linear signal transducer) and left ventricular (LV) PV measurements using an admittance catheter (ADVantage, Science, Canada). *In vitro* assessment of cardiac function was made in Langendorff-perfused hearts excised from mice at 5 and 9 weeks after sham or banding surgery and FFRs constructed over the range of 400–750 bpm.

2.4 Electrophysiological measurements of Na/K pump activity

Myocytes were isolated from hearts of sham and banded mice (from 5 to 9 weeks post-surgery) using conventional collagenase digestion techniques and Na/K pump current measured using whole-cell voltage clamp, as described previously.²² Cell capacitance was measured from the capacitance transient generated on application of a voltage step from the holding potential of –90 to –80 mV. Currents generated were recorded via an Axopatch 200A amplifier and a pClamp10 software (Molecular Devices, CA, USA).

2.5 Fluorescence measurements of intracellular Na and Ca

Intracellular Na was measured in quiescent and paced myocytes using 1,3-benzene-dicarboxylic acid, 4,4'-[1,4,10-trioxo-7,13-diazacyclopentadecane-7,13-diylbis(5-methoxy-6,12-benzofurandiyl)]bis-, tetrakis[acetoxymethyl] ester (SBFI) as previously described by Despa *et al.*²³ (see Supplementary

material online for details). Intracellular Ca and Na/Ca exchanger (NCX) activity were measured in paced myocytes using Fura-2 AM as described in Supplementary material online.

2.6 Immunoblotting

Hearts were harvested from banded and sham-operated animals 4 and 8 weeks after aortic banding surgery. Tissue was prepared, and western blots run, as described in Supplementary material online.

2.7 Statistical data analysis

Quantitative data are shown as means \pm standard error of the mean (SEM). Differences between experimental groups were tested by one-way or two-way ANOVA followed by a Bonferroni *post hoc* test or by paired or unpaired *t*-tests as appropriate. *P*-values of <0.05 were considered significant. Western blots that were quantitatively compared with multiple control and test samples were all run on the same resolving gel. The average densitometric value for the multiple control samples was defined as 1.0 and all individual bands (including the control samples) were normalized to this average—hence providing an SEM for control and test samples. In electrophysiological and fluorescence experiments, a number of cells from each isolation were studied; observations from a single-cell isolation were averaged and the *n*-value represents the number of hearts from which cells were isolated.

3. Results

3.1 Pressure overload-induced progressive cardiac remodelling and contractile dysfunction in C57BL/6J mice

The progression and characteristics of cardiac hypertrophy were confirmed in this model (see Supplementary material online, *Figure S1* and *Table S1*). A progressive cardiac remodelling was observed over the 8-week period such that LV mass/tibia length ratio increasing from 5.4 ± 0.18 ($n = 8$) in sham to 12.3 ± 0.65 ($n = 8$) in banded animals. This increase in LV mass was accompanied by decreases in ejection fraction, cardiac output, and velocity time integral (see Supplementary material online, *Figure S1*). Detailed analysis of echocardiographic data from sham and banded animals 4 and 8 weeks after surgery is shown in Supplementary material online, *Table S1*. Accompanying the increase in LV mass was a progressive systolic and diastolic dilation and contractile dysfunction.

3.2 LV hypertrophy did not change PLM/NKA ratio, but reduced PLM phosphorylation at Ser63 and 68

Western blot analyses were carried out on whole LV homogenates to determine changes in the expression of PLM, Na/K ATPase ($\alpha 1$ and $\alpha 2$ subunits), and PLM phosphorylation in response to aortic constriction. Pressure overload did not change $\alpha 1$ and $\alpha 2$ Na/K ATPase or PLM expression. Importantly, therefore, in this model, the PLM: $\alpha 1$ ratio was unaffected by hypertrophy (*Figure 1*). However, PLM phosphorylation at Ser63 and 68 was significantly reduced in banded mice (*Figure 1*). PLM phosphorylation at Ser69 was unaffected by pressure overload (mean data not shown). Hypophosphorylation of PLM could be expected to reduce Na/K pump function in the hypertrophied myocardium.

To test the hypothesis that this hypophosphorylation of PLM is preceded by a transient period of hyperphosphorylation (before sympathetic pathways down-regulate), we studied two further cohorts of mice banded for 3 and 14 days. *Figure 2A* and *B* shows that as early as 3 days there was no

detectable increase in PLM phosphorylation. In fact, PLM Ser68 phosphorylation was already decreased by 3 and 14 days post-banding, and there was a similar non-significant trend for an early decrease in Ser63 phosphorylation. The scatter in these data is large, possibly due to the varying and uncontrollable sympathetic stress associated with the recent surgery in both the sham and banded animals. *Figure 2A* and *B* shows NCX and SERCA2a expression at these early time-points. NCX was markedly up-regulated by 3 days, whereas SERCA2a clearly starts to decrease by Day 3 and is significantly down by Day 14.

3.3 NKA current density decreased in response to pressure overload

To investigate functional consequences of PLM hypophosphorylation, we measured Na/K pump current in ventricular myocytes isolated from banded and sham-operated mice. Cell capacitance was significantly increased by 67% in banded mice (*Figure 3A*). This compares with a 128% increase in heart weight/tibia length ratio, indicating that the increase in heart mass reflects not only the increase in myocyte size but also increases in non-myocyte components. Interestingly, as exemplified in *Figure 3B*, the absolute amount of pump current was greater in hypertrophied compared with sham-operated cells but, when normalized for cell size, pump current density was markedly reduced in hypertrophied cells (*Figure 3C*). There was a strong correlation between the level of hypertrophy and the extent of pump current inhibition (*Figure 3D*). Interestingly, this relationship, although shifted to higher pump current densities, is also seen within cells from sham-treated animals. That is, within the usual distribution of normal cell sizes isolated from control hearts, larger cells have lower pump current densities. The relationship between cell size and pump current density appears to be curvilinear and is steeper in control than hypertrophied cells (*Figure 3D*).

3.4 Contractile function in Langendorff hearts isolated from sham and banded mice

At a constant pacing rate of 550 bpm, hypertrophied hearts showed significantly reduced contractile function over 20 min of control perfusion (*Figure 3E*). The FFR for sham and banded hearts is shown in *Figure 3F*. In sham-operated hearts, this relationship was initially positive over the range 400–500 bpm before LV developed pressure (LVDP) declined at higher heart rates. In banded hearts, the FFR was entirely negative over the range of 400–750 bpm.

3.5 The basal cardiac phenotype of PLM^{3SA} mice

In this study, we have used the PLM^{3SA} mouse to examine the role of PLM phosphorylation in cardiac hypertrophy. Before banding these animals, however, we have undertaken a characterization of the basal phenotype. Supplementary material online, *Table S2* shows echocardiographic and PV catheter-based assessment of basal function in anaesthetized PLM^{3SA} mice and their WT littermates. Under basal conditions, there were no significant changes in the majority of contractile and other variables.

3.6 Na/K ATPase and PLM expression in hearts from PLM^{3SA} and WT mice

Figure 4 shows the expression of Na/K ATPase α subunits, PLM, and other ion translocating proteins in hearts from 14- to 16-week-old WT and PLM^{3SA} mice. In hearts from PLM^{3SA} mice, $\alpha 1$ subunit expression, although elevated, was not significantly increased while PLM

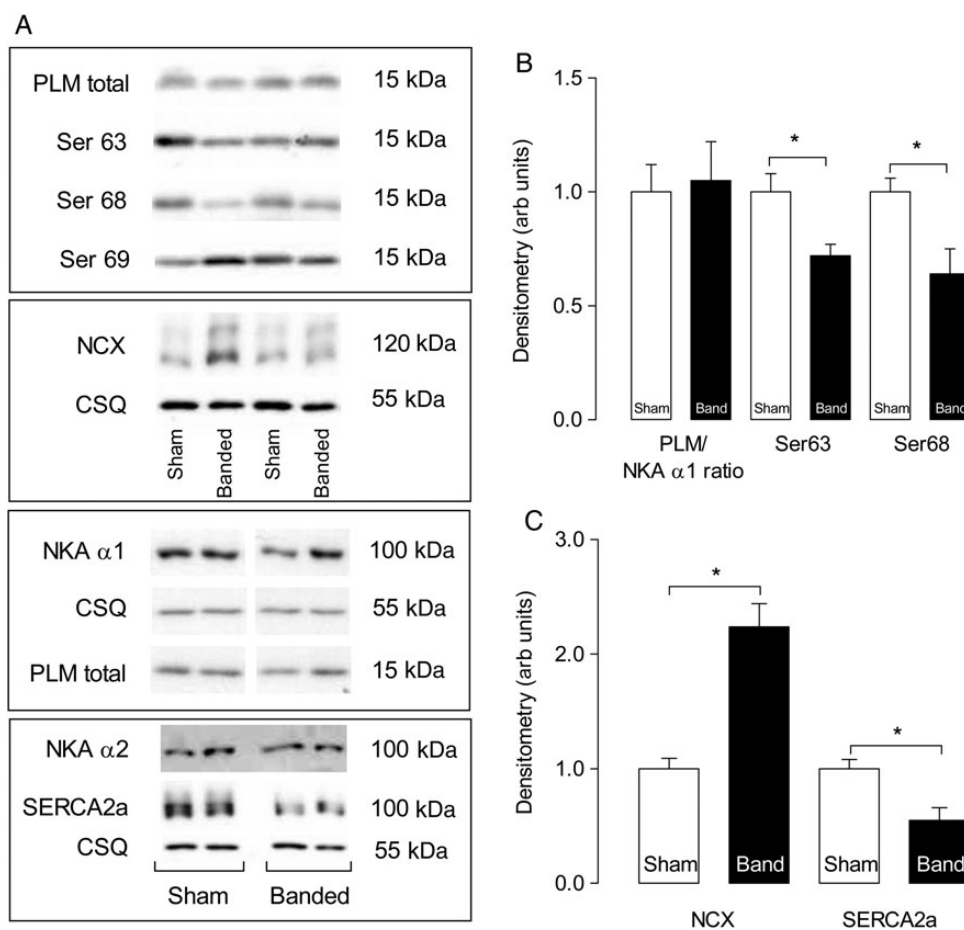


Figure 1 Protein expression and phosphorylation changes measured 8 weeks after aortic banding in C57BL/6J mice. Representative western blots (A) and quantitative changes in PLM/NKA α 1 ratio and PLM phosphorylation (B). PLM phosphorylation was normalized to total PLM. (C) Changes in the expression of NCX and SERCA2a in sham and banded hearts. PLM, phospholemman; NKA α 1, Na/K ATPase α 1 subunit; CSQ, calsequestrin; NCX, sodium–calcium exchanger; SERCA2a, sarco/endoplasmic reticulum Ca ATPase type 2a. In A, the top and bottom panels show images of the same samples cropped from different gels. All other panels were each run on a single gel and images cropped within a gel (CSQ or total PLM was used as loading controls). Data in B and C are mean \pm SEM, * P < 0.05; n = 6–11 (sham) and n = 6–10 (banded).

expression was significantly decreased. This combination of slightly increased α 1 and decreased PLM resulted in a significant decrease in the PLM/ α 1 ratio (Figure 4C). Figure 4B also confirms that antibodies to PLM phosphorylated at Ser63, 68, and 69 were unable to detect phosphorylated PLM in PLM^{35A} mice.

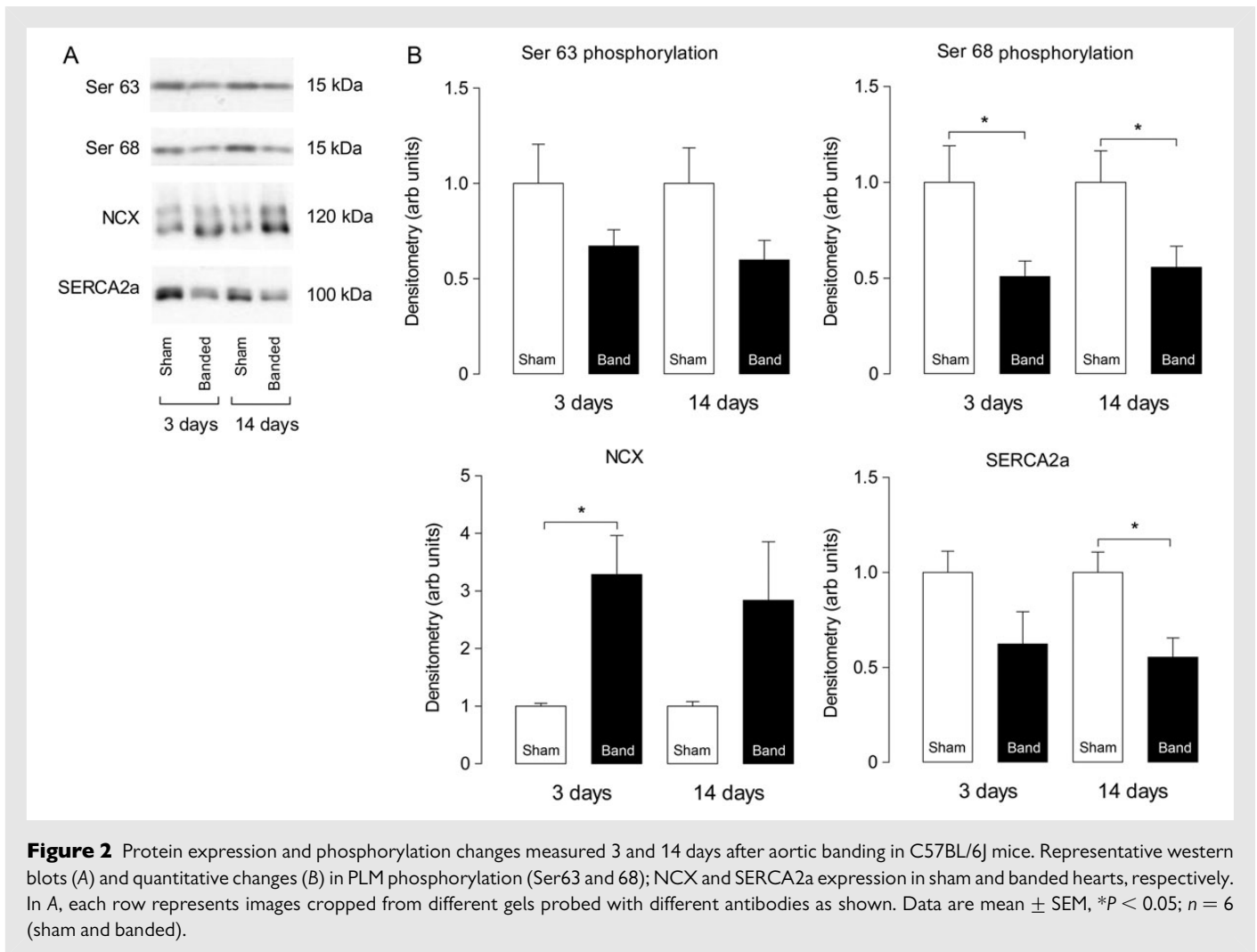
The Na/K ATPase α 2 subunit expression was increased in PLM^{35A} hearts (Figure 4D). This, combined with the decrease in PLM expression, results in a significant decrease in PLM/ α 2 ratio. Under basal conditions, this increase in the relative expression of both α 1 and α 2 relative to PLM may reflect an adaptive response to the inhibitory effect of unphosphorylated PLM on Na/K transport. This adaptation appears not to be confined to the Na/K pump complex, but significantly higher levels NCX were also measured in PLM^{35A} hearts (SERCA2a levels were unaffected) (Figure 4E). While such adaptive changes may allow normal ion regulation under basal unstimulated conditions, the mutation of PLMs phosphorylation sites should render the Na/K pump unresponsive to PKA activation or β -receptor stimulation. *In vivo* where tonic autonomic control may be important, this may explain the normal contractility seen *in vivo* despite significant difference *in vitro*.

3.7 Basal Na/K pump current in PLM^{35A} mice and response to forskolin

We have previously shown that basal Na/K pump current was similar in both genotypes, but while WT myocytes responded to forskolin (50 μ M/L), PLM^{35A} myocytes were unresponsive.²⁴ Under unstimulated conditions, PLM in WT myocytes is up to 40% phosphorylated.¹⁰ This basal phosphorylation is obviously absent in PLM^{35A} mice. It therefore appears that, in unstimulated PLM^{35A} myocytes, the inhibitory effect of expressing unphosphorylatable PLM is offset by the compensatory decrease in PLM expression and increase in alpha subunit expression (Figure 4). Baseline pump current is therefore maintained at normal levels, but is subsequently unable to increase when exposed to forskolin or indeed any other kinase-mediated stimulus.

3.8 Aortic constriction and global and cellular remodelling in PLM^{35A} mice

If the hypophosphorylation of PLM is not causally involved in the progression of hypertrophy, and simply 'reports' on the down-regulation of β -adrenergic signalling pathways, then PLM^{35A} and WT littermates



should be equally vulnerable to hypertrophy. On the contrary, if PLM hypophosphorylation is causally involved in the progression of hypertrophy, then remodelling following aortic constriction may be expected to be more severe in the PLM^{3SA} mouse (where PLM phosphorylation is not just limited it is completely prevented).

Figure 5 shows that the hypertrophic response to pressure overload was markedly increased in PLM^{3SA} mice compared with WT animals. In WT mice 8 weeks after surgery, left ventricle/body weight (LV/Bw) ratio increased by 56% from 4.1 ± 0.06 mg/g in shams to 6.4 ± 0.65 mg/g in banded animals. In PLM^{3SA} mice, however, this hypertrophic response was substantially exacerbated with LV/Bw ratio, increasing by 142% from 3.8 ± 0.12 to 9.2 ± 1.08 mg/g. This markedly increased remodelling was accompanied by significantly more dilation both in systole and in diastole and an exacerbation of the banding-induced fall in ejection fraction. More detailed analysis of the echocardiographic measurements is shown in Supplementary material online, Table S3. All ejection-phase indices were significantly lower in PLM^{3SA} mice after aortic constriction.

LV function was also assessed by a catheter-based PV analysis approach in closed-chest mice (see Supplementary material online, Table S3). No differences were observed in sham-treated groups, but banded mice showed demonstrable LV dysfunction. After aortic constriction, end-systolic pressure was elevated to the same extent in WT and PLM^{3SA} mice, suggesting that both genotypes were subjected

to the same level of pressure overload in response to aortic banding. This was confirmed by post-mortem observation of the RLA which was identical in banded mice of both genotypes (data not shown). Any differences between WT and PLM^{3SA} mice were, therefore, not attributable to changes in the severity of the pressure overload. Interestingly, the major difference between PLM^{3SA} banded and WT banded mice was a more profound slowing of relaxation (dp/dt min) and the LV relaxation time-constant (T_{Weiss}). These data are consistent with the echocardiographic measurements and indicate a substantial degree of systolic and diastolic dysfunction in PLM^{3SA} mice after aortic constriction.

3.9 Na/K pump current and intracellular Na in hypertrophied PLM^{3SA} myocytes

To investigate the relevance of PLM-dependent regulation of Na/K pump activity in cardiac remodelling, we measured Na/K pump current and intracellular Na in myocytes from banded and sham-operated mice of both genotypes. In addition, Na/K pump and PLM expression was measured in LV homogenates from WT and PLM^{3SA} hearts. Pressure overload did not significantly change $\alpha 1$ and $\alpha 2$ Na/K ATPase or PLM expression and PLM to Na/K ATPase $\alpha 1$ subunits (NKA; $\alpha 1$ and $\alpha 2$ subunits) ratios in WT and PLM^{3SA} mice (see Supplementary material online, Figure S2). Consistent with the profoundly increased cardiac remodelling in the PLM^{3SA} mice, the hypertrophy-induced increase in cell size was

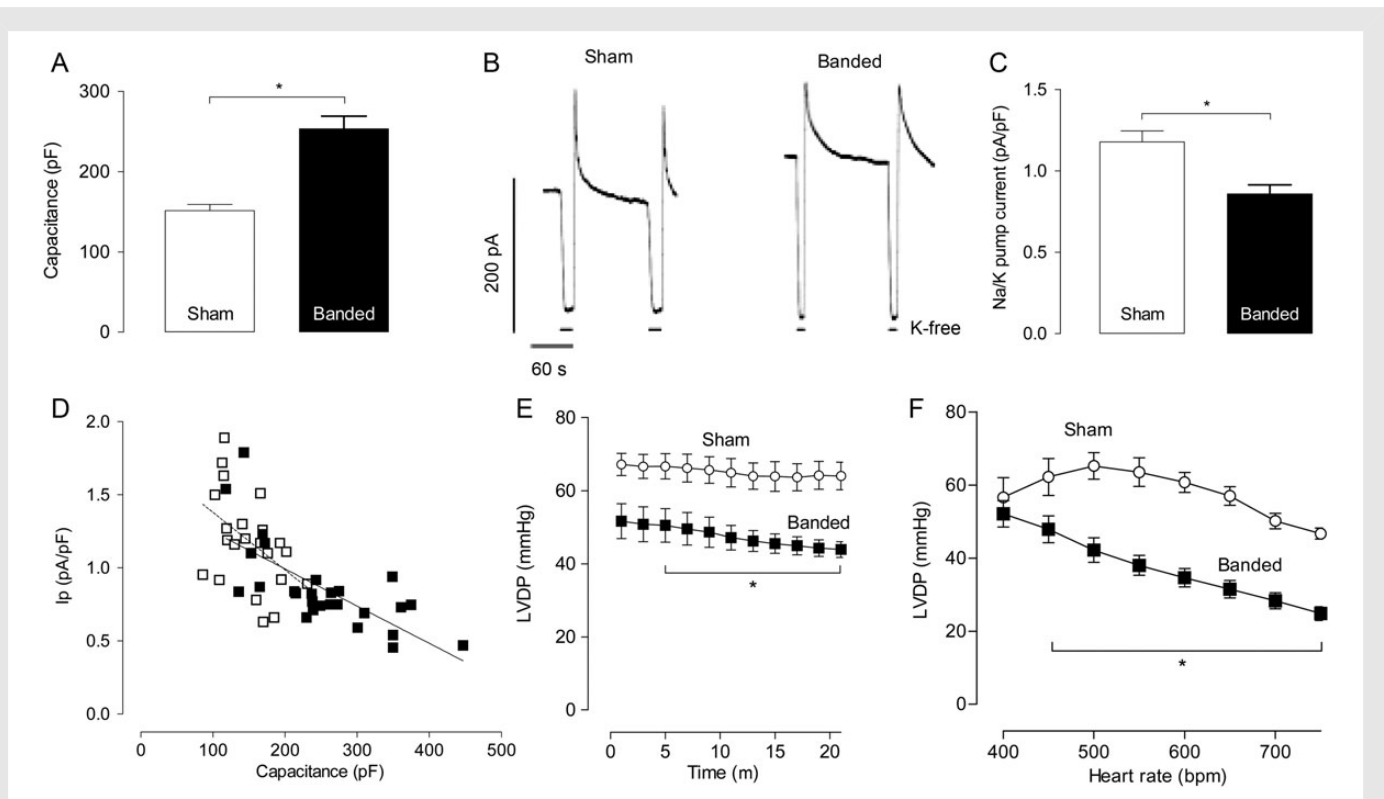


Figure 3 Na/K pump current measurements in myocytes and cardiac function in Langendorff hearts isolated from sham and banded C57BL/6J mice. (A) Cell capacitance showing significant myocyte hypertrophy in the banded group, (B) original traces showing example Na/K pump current recordings (the downward deflections represent periods of K-free superfusion), (C) mean pump current density in cells isolated from sham and banded hearts normalized to cell capacitance, and (D) relationship between pump current density and cell size (capacitance). The exponential decay curve was fitted to all points using the equation $Y = (Y_0 - \text{Plateau}) \times \exp(-K \times X) + \text{Plateau}$, where K is the 'rate' constant. (E) LVDP over a 20 min period of aerobic perfusion (pacing rate 550 bpm). (F) LV pressure–frequency relationship in sham and banded hearts. [Numerical data are mean \pm SEM, A and C, 23 cells from eight sham mice ($n = 8$) and 27 cells from nine banded mice ($n = 9$). In E and F, comparison between groups (sham and banded, $n = 6/\text{group}$) was performed using two-way ANOVA, repeated measures, * $P < 0.05$].

significantly greater in myocytes from banded PLM^{3SA} vs. banded WT mice (Figure 6A). The hypertrophy-induced reduction in Na/K pump current density was also more than doubled from a 24% reduction in banded WT myocytes to a 57% reduction in banded PLM^{3SA} myocytes (Figure 6B). The relationship between Na/K pump current density and cell size is shown in Figure 6C. As previously shown (Figure 3D), this relationship is shifted to the right and is shallower in banded myocytes. In PLM^{3SA} myocytes, the further reduction in pump current density was seen across all cell sizes and is again shallow and right-shifted vs. shams. Figure 6E shows intracellular Na measured in quiescent myocytes from sham and banded PLM^{3SA} mice and their WT littermates. In unpaced cells, intracellular Na was not significantly higher in PLM^{3SA} vs. WT shams, but was significantly elevated in both genotypes in response to banding. While this elevation in intracellular Na in PLM^{3SA} myocytes was not significantly higher than that in WT myocytes, it is important to note that these measurements were made in quiescent cells. Indeed, in the presence of field stimulation (1 Hz), we observed a significant increase in intracellular Na in PLM^{3SA} myocytes compared with WT (Figure 7A).

3.10 NCX in PLM^{3SA} myocytes

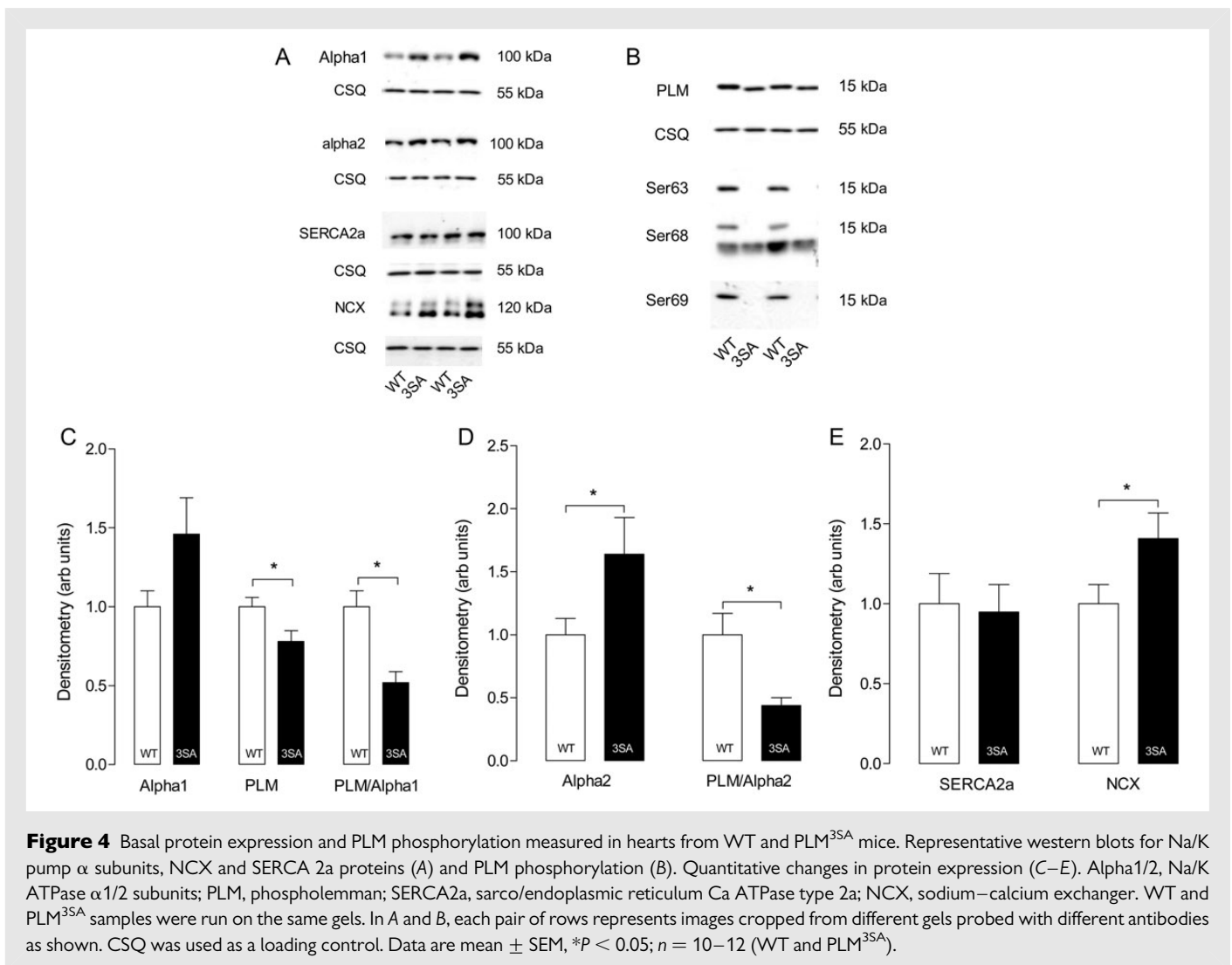
The elevation of intracellular Na in PLM^{3SA} myocytes will influence the activity of NCX. In addition, PLM has been shown to directly interact with NCX itself with phosphorylated PLM inhibiting exchanger

activity.^{25,26} We therefore investigated NCX activity in paced myocytes isolated from WT and PLM^{3SA} mice. Figure 7A shows that pacing significantly elevated intracellular Na in both WT and PLM^{3SA} cells (cf. Figure 6E). The rate of decline in the Ca transient induced by the rapid and maintained application of caffeine was used as an index of forward-mode NCX activity in both genotypes (Figure 7B and C). Figure 7D shows that the rate constant of Ca recovery was significantly prolonged in PLM^{3SA} myocytes, consistent with the elevation of intracellular Na shown in Figure 7A. Application of caffeine in the presence of Na-free Tyrode prevents forward-mode NCX and induces reverse-mode NCX increasing intracellular Ca (Figure 7B).

4. Discussion

The principle finding of this study is that decreased phosphorylation of the Na/K ATPase regulatory protein PLM reduces Na/K pump activity and exacerbates Na overload, contractile dysfunction, and adverse remodeling following aortic constriction in mice. The direct contribution of this Na overload to contractile (particularly diastolic) dysfunction and remodeling suggests a novel therapeutic target for the treatment of HF.

In C57BL/6J WT mice, cardiac PLM is hypophosphorylated in response to aortic constriction. This is associated with a reduction in Na/K pump function, as assessed by voltage-clamping, and an increase

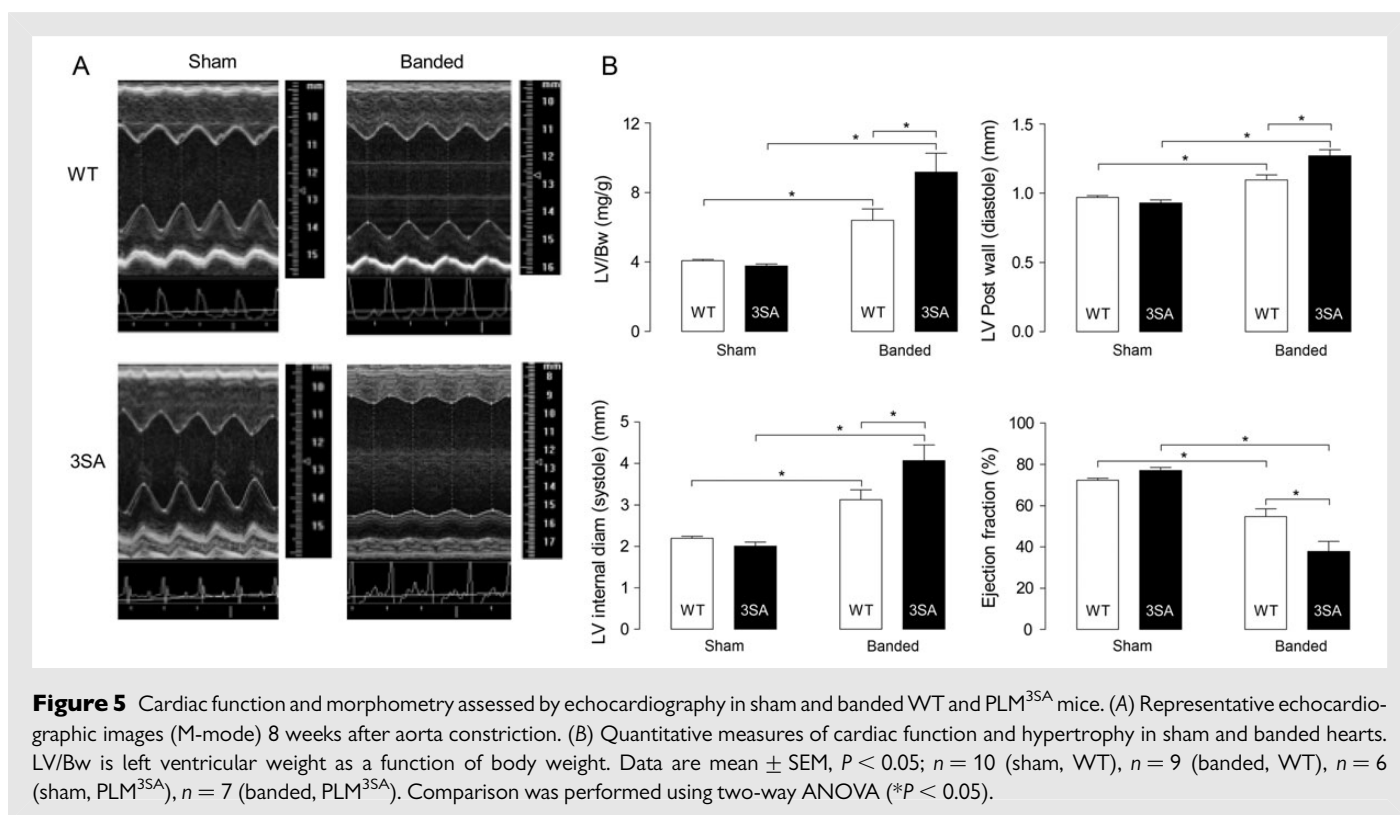


in intracellular Na. The hypophosphorylation of PLM was mirrored by a similar hypophosphorylation of another PKA substrate PLB (not shown). Although the contribution of PLB hypophosphorylation has been extensively studied, the relationship between PLM and the Na/K ATPase in hypertrophy and failure remains relatively poorly defined. As has previously been extensively reported, we find that SERCA2a expression is down-regulated and NCX significantly up-regulated in the hypertrophied heart. PLM and Na/K ATPase expression, however, remained essentially unchanged such that the ratio of PLM/Na/K ATPase α 1 subunit was constant over the 8-week period of cardiac remodelling.

These observations are consistent with our studies in human HF samples, where PLM was similarly hypophosphorylated.²⁰ In this study, hypophosphorylation was attributed to a down-regulation of inhibitor-1 and increased PP-1 phosphatase activity. Although we hypothesized that this hypophosphorylation may be preceded by a period of hyperphosphorylation (prior to β -adrenoceptor pathway down-regulation), no evidence was found for this at two earlier time-points 3 or 14 days after banding. Since the cardiac dysfunction induced by acute sympathetic activation, such as in Takotsubo cardiomyopathy, resolves within days or weeks,²⁷ it is possible that β -adrenergic signalling down-regulation occurs early after banding despite chronic

elevation of circulating catecholamines. Soltysinska et al.²⁸ have reported chronic down-regulation of β -signalling after 16 days of isoproterenol infusion, but did not look at earlier time-points. We attempted to investigate time-points earlier than 3 days post-banding (not shown), but the stress of the surgery in both banded and sham animals was so profound and variable that the data were too variable to be useful. These data therefore suggest that if there is a transient peak in sympathetically mediated phosphorylation of PKA substrates early after aortic constriction, it occurs within 3 days and this signalling pathway rapidly down-regulates leading to a maintained hypophosphorylation of PLM and PLB.

In this model, in WT mice, expression of Na/K ATPase α 1 subunit and PLM was unchanged and hence the decrease in Na/K pump current measured in isolated cells we attribute to the dephosphorylation of PLM. This decrease in Na/K pump current was curvilinearly associated with the extent of myocyte hypertrophy (the larger the cell the smaller the pump current density) and with an increase in intracellular Na. In these studies, total pump current was measured in voltage-clamped myocytes in a ruptured-patch mode where the subsarcolemmal Na concentration may not exactly match pipette Na. Pipette Na in these experiments was fixed at 25 mmol/L to allow the detection of changes in pump V_{max} and K_m for Na. However, in hypertrophied cells, diffusional access



to the subsarcolemmal space may be more limited than in control cells and it is possible that the decline in pump current in the larger more hypertrophied cells could also reflect changes in subsarcolemmal Na due to limitations in cell dialysis.

In the introduction to this paper, we raise the possibility that the hypophosphorylation of PLM in the hypertrophied heart, as confirmed in this study, may simply be a bystander effect—reporting on the prevailing state of the β -adrenergic signalling pathway without causally affecting the progression of the disease. In this study, we have used the PLM^{3SA} mouse to address this issue. Since PLM in this mouse is rendered unphosphorylatable, development of the disease should be unaffected if it is simply a reporter and exacerbated if it is causally involved. Data presented here clearly indicate that the complete prevention of PLM phosphorylation at Ser63, 68, and 69 markedly exacerbates remodelling. Hearts from PLM^{3SA} respond to the same pressure overload as WT hearts with a markedly more severe hypertrophy, increased contractile dysfunction, and more depressed Na/K pump function. This demonstrates that the extent of PLM phosphorylation influences the development of the disease and is not simply passively reporting on the state of PKA or PKC activation.

In addition to the mutations in PLM, the PLM^{3SA} mice also have adaptive changes in NCX that could influence both E–C coupling and the response to a hypertrophic stimulus. The overexpression of NCX seen in these mice is, however, unlikely to exacerbate cardiac hypertrophy. Adachi-Akahane *et al.*²⁹ showed that mice overexpressing NCX show no evidence of cardiac hypertrophy. So, it seems unlikely that the increased vulnerability of the PLM^{3SA} heart to hypertrophic remodelling and contractile dysfunction is attributable to these relatively small adaptive changes in NCX.

In addition to changes in baseline NCX expression, PLM^{3SA} myocytes also show a significant increase in the expression of the $\alpha 2$ isoform of the Na/K ATPase. Correll *et al.*³⁰ have suggested that the $\alpha 2$ isoform is 'less

regulated by PLM' and provides a mechanism by which $\alpha 2$ can efficiently couple to NCX, particularly in pathology, to maintain ion (and particularly Ca) regulation. In their study, they show that the 'forced' overexpression of $\alpha 2$ leads to a reduction in PLM and PLM phosphorylation. It seems possible that the reciprocal is true in our study—that is, the 'forced' expression of unphosphorylated PLM leads to increased $\alpha 2$ expression. This compensatory change may allow Na regulation in microdomains relevant to E–C coupling to be preserved despite a bulk rise in cytoplasmic Na. In normal (unbanded) hearts, this increase in $\alpha 2$ is significant with PLM^{3SA} hearts having more $\alpha 2$ protein and a lower PLM/ $\alpha 2$ ratio (Figure 4). In banded PLM^{3SA} hearts, this relationship seems to persist but, with the increased variability introduced by hypertrophy, fibrosis, etc., statistical significance is lost (see Supplementary material online, Figure S2).

PLM, in addition to interacting with Na/K ATPase, has also been shown to directly modulate NCX independent of changes in ion gradients.²⁵ It is therefore possible that the pro-hypertrophic effects of the PLM^{3SA} mutation could be attributable to a direct interaction between the mutated PLM and NCX. Studies from Cheung *et al.*²⁶ have shown that phosphorylated PLM inhibits NCX. As WT PLM is substantially phosphorylated at baseline (Figure 4B), it is possible that the 3SA mutant would effectively stimulate NCX when compared with WT controls. However, this was not seen. In the present study, we found that NCX activity was significantly reduced in PLM^{3SA} myocytes compared with the WT cells (Figure 7), despite a significant increase in NCX expression in the PLM^{3SA} hearts (Figure 4). This is consistent with the study of Song *et al.*³¹ who reported that unphosphorylated PLM (four potential phosphorylation sites mutated to alanines in a 3S1T mutant) transfected into rat myocytes was without effect on contraction and presumably also without effect on NCX. The reduction in forward-mode NCX activity in PLM^{3SA} myocytes is therefore most likely to be due to the increased [Na]_i observed in 3SA cells and not to a direct

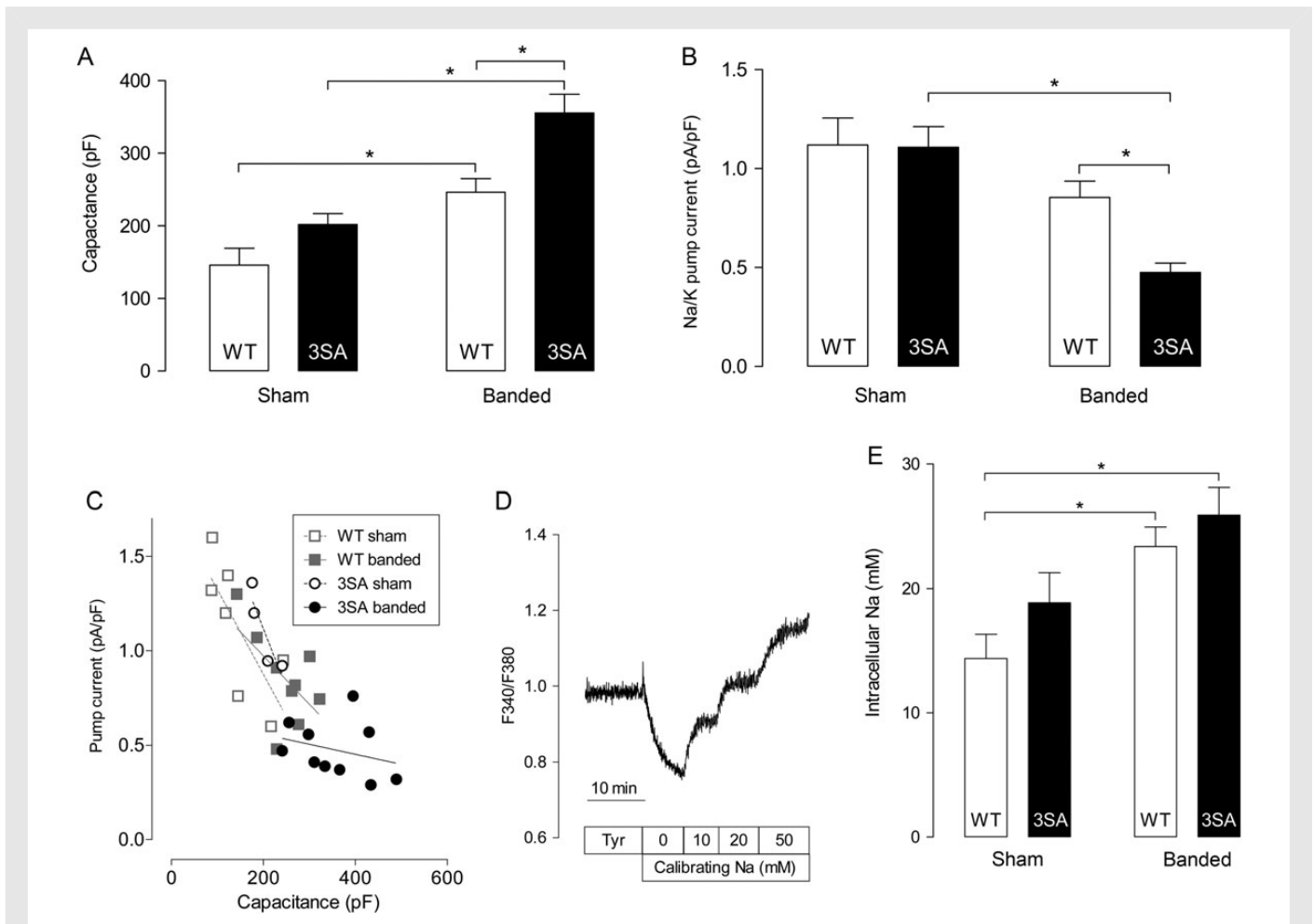


Figure 6 Na/K pump current and intracellular Na measurements in myocytes isolated from sham and banded WT and PLM^{35A} hearts. (A) Cell capacitance showing significantly increased myocyte hypertrophy in the PLM^{35A} banded group, (B) mean pump current density in cells isolated from sham and banded WT and PLM^{35A} hearts normalized to cell capacitance, and (C) relationship between pump current density and cell size (capacitance). Each individual dataset is fitted with a linear regression. (D) Example trace showing calibration of SBFI fluorescence, and (E) mean intracellular Na concentrations in sham and banded myocytes from WT and PLM^{35A} mice. Data in A, B, and E are mean \pm SEM; in A and B, seven cells from four sham WT ($n = 4$), nine cells from five banded WT ($n = 5$), four cells from four sham PLM^{35A} ($n = 4$), and 10 cells from five banded PLM^{35A} hearts ($n = 5$); in E, 15 cells from five sham WT ($n = 5$), 12 cells from seven banded WT ($n = 7$), 24 cells from seven sham PLM^{35A} ($n = 7$), and 27 cells from five banded PLM^{35A} hearts ($n = 5$). Comparison was performed using two-way ANOVA ($*P < 0.05$).

interaction between the mutated PLM and NCX. This increase in Na may also explain the negative FFR seen in Langendorff-perfused hearts from banded animals as this has also previously been associated with an elevation of intracellular Na in normal rodent hearts³² and hypertrophied/failing human hearts.⁷

The observation that the hypophosphorylation of PLM, and associated Na/K pump inhibition, exacerbates cardiac remodelling and dysfunction raises the possibility that conversely, the preservation of PLM phosphorylation and/or Na/K pump stimulation may be therapeutically useful in HF. In the context of HF, superficially, there appears to be an interesting historical contradiction in this hypothesis. Na/K pump *inhibition*, in the form of digitalis, has been used to treat HF for over 200 years. Digitalis, however, appears to provide symptomatic relief rather than a positive effect on long-term prognosis. In the largest trial of its kind, the Digitalis Investigation Group (DIG) showed digoxin-reduced hospitalization due to worsening HF symptoms, but had no long-term effect on mortality.³³ Conversely, there is a remarkable correlation between

the ability of wide variety of agents to *stimulate* the Na/K pump and a beneficial outcome of many clinical trials for HF.³⁴ ACE inhibitors, ARBs, aldosterone antagonists, NO donors, and insulin are all known to stimulate the Na/K ATPase and, in clinical trials, these agents have all been shown to be protective.³⁴ The exception to this is β -blockers (which might be expected to limit pump stimulation), but these are clearly beneficial. Incongruously, it is, however, now generally accepted that β -blockers can somehow preserve cellular adrenergic signalling perhaps by preventing β -receptor down-regulation.¹⁶ Thus, the weight of evidence suggests that a selective means of increasing Na/K pump activity that can increase Na extrusion *without* inducing PKA activation and Ca overload is required. Such a therapy may complement existing therapies such as β -blockade. Limiting Na overload by selective pump stimulation may also be beneficial in diseases other than HF such as hypertension, angina, and arrhythmias (especially catecholaminergic polymorphic ventricular tachycardia) where Na overload is known to be causally involved.³⁵

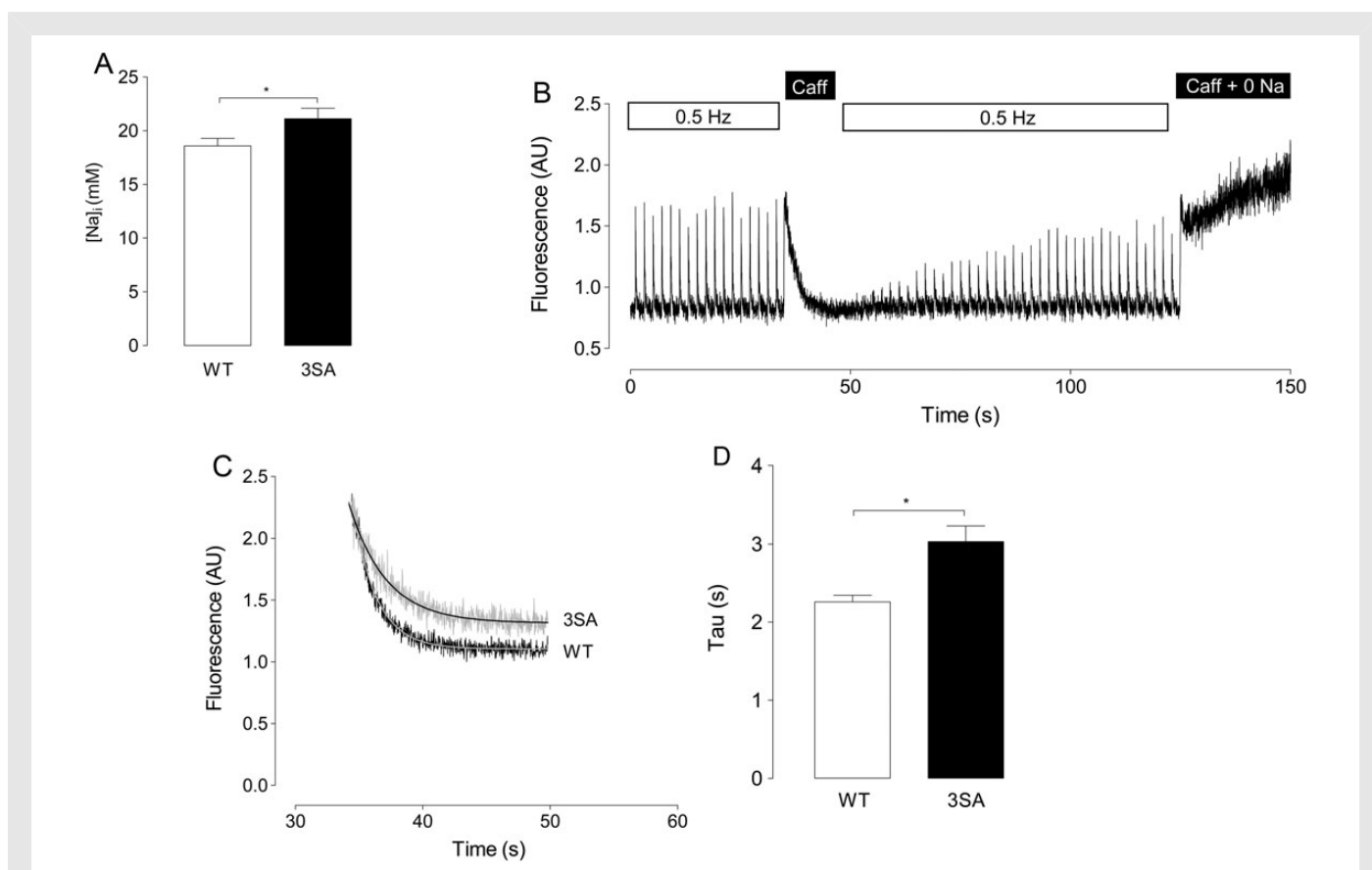


Figure 7 Intracellular Na and NCX activity measurements in paced myocytes isolated from WT and PLM^{3SA} hearts. (A) Mean intracellular Na concentrations in myocytes from WT and PLM^{3SA} mice paced at 1 Hz, (B) example trace showing Ca transients (0.5 Hz) and the caffeine-induced Ca release from the SR used to calculate the NCX activity, (C) original traces of caffeine-induced Ca transient decay in myocytes isolated from WT and PLM^{3SA} hearts (a mono-exponential function was fitted to the curve the time-constant of which is assumed to be largely inversely related to NCX activity), and (D) forward-mode NCX activity assessed by the mean time-constants of the caffeine-induced Ca transient decay in cells isolated from WT and PLM^{3SA} mice. Data in A and D are mean ± SEM; in A, 46 cells from three WT hearts ($n = 3$) and 34 cells from three PLM^{3SA} hearts ($n = 3$); in D, 29 cells from five WT hearts ($n = 5$) and 30 cells from five PLM^{3SA} hearts ($n = 5$), * $P < 0.05$.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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