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# Phospholemman phosphorylation regulates vascular tone, blood pressure and hypertension in mice and man

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**Short title:** Boguslavskyi - Na/K ATPase and blood pressure control

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**ABSTRACT**

**Background:** While it has long been recognized that smooth muscle Na/K ATPase (NKA) modulates vascular tone and blood pressure (BP), the role of its accessory protein phopholemman (PLM) has not been characterized. The aim of this study was to test the hypothesis that PLM phosphorylation regulates vascular tone *in vitro* and this mechanism plays an important role in modulation of vascular function and BP in experimental models *in vivo* and in man.

**Methods:** *Mouse studies:* PLM knock-in mice ($PLM^{3SA}$), in which PLM is rendered unphosphorylatable, were used to assess the role of PLM phosphorylation *in vitro* in aortic and mesenteric vessels using wire myography and membrane potential measurements. *In vivo* BP and regional blood flow were assessed using Doppler flow and telemetry in young (14-16 weeks) and old (57-60 weeks) wild-type (WT) and transgenic mice. *Human studies:* We searched human genomic databases for mutations in PLM in the region of the phosphorylation sites and performed analyses within two human data cohorts (UK Biobank and GoDARTS) to assess the impact of an identified SNP on BP. This SNP was expressed in HEK cells and its effect on PLM phosphorylation determined using Western Blotting.

**Results:** PLM phosphorylation at Ser63 and Ser68 limited vascular constriction in response to phenylephrine. This effect was blocked by ouabain. Prevention of PLM phosphorylation in the $PLM^{3SA}$ mouse profoundly enhanced vascular responses to PE both *in vitro* and *in vivo*. In ageing WT mice PLM was hypophosphorylated and this correlated with the development of ageing-induced essential hypertension. In man we identified a non-synonymous coding variant, single nucleotide polymorphism rs61753924, which causes the substitution R70C in PLM. In HEK cells the R70C mutation prevented PLM phosphorylation at Ser68. This variant's rare allele is significantly associated with increased BP in middle-aged men.

**Conclusions:** These studies demonstrate the importance of PLM phosphorylation in the regulation of vascular tone and BP and suggest a novel mechanism, and therapeutic target, for ageing-induced essential hypertension in man.

**CLINICAL PERSPECTIVE**

**What is new?**

- Despite many previous studies implicating smooth muscle Na/K ATPase and endogenous cardiotonic steroids in hypertension, this is the first study demonstrating a role for its principle regulatory accessory protein phospholemman.
- Phosphorylation of phospholemman profoundly influences vascular tone and blood pressure regulation in mice and its hypophosphorylation is associated with ageing-induced essential hypertension.
- In man, a single nucleotide polymorphism was identified in phospholemman exon 10 that results in an R70C mutation that *in vitro* leads to phospholemman hypophosphorylation and, in two human cohorts (UK Biobank and GoDARTS), is associated with a significant elevation of blood pressure in middle-aged men.

**Clinical implications**

- Interventions that prevent PLM dephosphorylation and Na/K ATPase down-regulation may provide new therapeutic approaches to treatment of essential hypertension.
- Existing interventions that prevent down-regulation of signalling pathways that terminate in PLM phosphorylation (for example beta blockers) may in part exert their beneficial antihypertensive effect through this mechanism.
- The age and sex-dependence of these observations needs further investigation.

**INTRODUCTION**

The Na/K ATPase is ubiquitously expressed and maintains the Na and K trans-membrane gradients essential for a plethora of cell functions including electrical excitability, secondary active transport and cell signaling.[1-3]

In vascular smooth muscle cells (VSMCs) the Na/K ATPase (specifically the $\alpha 2$ isoform) plays an important role in modulating VSMC tone and blood pressure (BP).[1,2,4-7] The hypertensive effects of Na/K pump inhibition with both exogenous and endogenous cardiotonic steroids (CTS) have long been recognized.[8-10] Agents that stimulate the pump are hypotensive, while agents that inhibit the pump (and specifically the $\alpha 2$ subunit in VSM) are hypertensive.[8,9,11] Endogenous CTS, for example, play a role in setting BP[12] and exogenous ouabain completely blocks classical acetylcholine-induced NO-mediated vasodilation[13] and substantially blocks reactive hyperaemia[14] - suggesting that Na/K ATPase activity not only influences vascular contractility but is also central to the action of endogenous vasodilators.[4,15-19]

Despite overwhelming evidence for a role of the Na/K ATPase in regulation of smooth muscle tone and BP, surprising little is known about the role of phospholemman (PLM), the muscle-specific regulator of Na/K ATPase, in modulating BP and even less is known about its possible contribution to hypertension. PLM (FXYD1) is a member of the FXYD family of Na/K ATPase modulatory proteins and in cardiac muscle links cellular signaling pathways (specifically PKC and PKA) to Na/K pump activation.[20] In heart, phosphorylation of PLM disinhibits the Na/K ATPase by lowering its $K_m$ for Na and by increasing $V_{max}$ and hence increases Na efflux in response to hormonal stimulation and heart rate elevation.[21-23]

While PLM is expressed in VSM, its role in regulating Na/K ATPase in this tissue has not been established and the limited literature is incomplete and contradictory.[24,25,26] Thus, while there is limited evidence that PLM may regulate Na/K ATPase in VSM, the physiological relevance of this is far from certain and its role in the control of BP has yet to be demonstrated. The primary aim of this study

was, therefore, to test the hypothesis that PLM phosphorylation regulates vascular tone *in vitro* and this mechanism plays an important role in modulation of vascular function and BP *in vivo*.

## METHODS

An expanded methods section is available in the Data Supplement. The authors declare that all supporting data are available within the article and online Data Supplement. Access to source patient datasets is available to suitably qualified researchers through UKBB (https://www.ukbiobank.ac.uk) and GoDARTS (https://godarts.org).

### Animals and PLM$^{3SA}$ 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 King's College London Ethics Review Board. Male PLM$^{3SA}$ knock-in mice or their WT littermates (13-16 weeks of age (young) or 56-60 weeks of age (old)), were used in the majority of studies. A small number of studies used male Wistar rats (250g) (Charles River, UK).

### Myography

Vascular rings were isolated from thoracic aortae of WT and PLM$^{3SA}$ mice, or rats, mounted in a tension myograph (Danish Myo Technology), optimally stretched, endothelial integrity tested (with acetylcholine) and concentration-response curves to PE and the thromboxane $A_2$ mimetic U46619 constructed in the presence or absence of ouabain. Changes in isometric tension were sampled and recorded at 100Hz using a PowerLab and LabChart software (AD Instruments, New Zealand).

#### *In vivo hemodynamic assessment in mice:*

Central arterial BP was measured in anesthetized mice via a pressure tipped catheter (1.2F, Transonic) inserted retrogradely into the ascending aorta via the right common carotid artery. The left jugular vein was cannulated for PE infusion. Surface ECG was continuously recorded. Augmentation index (AI)

was calculated as previously described.[27] In separate experiments, non-invasive laser Doppler imaging (MoorLDI2 model; Moor Instruments Inc., Wilmington, USA) was used to assess hind-limb blood flow. Continuous hemodynamic measurements in conscious mice were performed by telemetry as described previously.[28] Cardiac structure and function were assessed by 2D echocardiography.[29]

**Immunoblotting:**

Thoracic aortae or mesenteric vessels were harvested from PLM$^{3SA}$ and WT mice, cleaned of fat and connective tissue, cut into 3-4 pieces and incubated at 37$^{o}$C in gassed Krebs solution or Krebs plus PE (10µmol/L), U46619 (1µmol/L) or spNONO (100µmol/L). The tissue was snap frozen and stored at -80$^{o}$C. Tissue homogenates (4%w/v) were size-fractionated on SDS-PAGE gels (10-15%) and processed for Western bloting as previously described.[29] Alpha smooth muscle actin was used as a loading control. Signals from PLM$^{3SA}$ samples or treated WT samples were normalized to signals from control WT samples on the same gels or, for PLM phosphorylation, to total PLM. Details of antibodies, procedures and quantification can be found in the Data Supplement.

**Membrane potential measurements:**

Membrane potential was measured in 2mm segments of third-order mesenteric arteries mounted in a Mulvany-Halpern wire myograph (model 400A, Danish Myo Technology, Denmark) using sharp glass microelectrodes backfilled with 2mol/L KCl (tip resistances ~100MΩ). Tension was simultaneously recorded.[30,31]

**Genomic and phenotypic screening in patient populations**

We searched human genomic databases for mutations in PLM in the region of the phosphorylation sites. rs61753924 is a single nucleotide polymorphism (SNP) at 35,633,635 base pairs position (build hg19/37) on chromosome 19, in PLM exon 10, a C to T transition in position 1 of the codon encoding arginine 70 that generates a non-synonymous variant with the amino acid substitution R70C. In order to assess the impact of this SNP on BP, we analysed this SNP within two different human cohorts: UK

Biobank (UKBB)[32] and Genetics Of Diabetes Audit and Research in Tayside (GoDARTS).[33,34] Details of the cohorts and the analysis can be found in the Data Supplement.

**Statistics:**

Data are shown as means±standard error of the mean (SEM). Details of the analyses used can be found in the data supplement.

## RESULTS

### *Prevention of PLM phosphorylation potentiates phenylephrine-induced vasoconstriction*

Figure 1A shows the dose-dependent constriction of isolated aortic rings to phenylephrine (PE) was potentiated in tissues isolated from PLM$^{3SA}$ mice. The response of PLM$^{3SA}$ tissue was unaffected by ouabain but inhibition of Na/K ATPase in WT tissue rendered the WT hyper-responsive to PE and similar to PLM$^{3SA}$ (Figure 2B).

While PE-induced constriction was profoundly different between genotypes, and this difference was Na/K ATPase dependent, no differences were seen between genotypes in response to U46619 (Figure 1C). While U46619 interestingly also induced PLM phosphorylation at Sers63 and 68 (Figure 1F), this did not limit maximal constriction in WT aortae suggesting that, unlike PE-induced constriction, high concentrations of U46619 (0.1-30µmol/L) mediate their constrictor effects via cellular mechanisms that are largely unaffected by PLM phosphorylation and Na/K ATPase activity. This is unsurprising as at these concentrations U46619 has been shown to potently inhibit the Na/K ATPase.[35]

In parallel experiments, Western blot analysis (Figure 1D-F) showed that PE (10µmol/L) and U46619 (1µMmol/L) induced substantial PLM phosphorylation at Ser63 (PE) and Ser63 and Ser68 (U46619) in WT aortae. Expression of PLM, and both $\alpha$1 and $\alpha$2 subunits of the Na/K ATPase in aortic smooth muscle, were unaffected by genotype (see Supplement Figure 1).

A similar disparity between the role of Na/K ATPase in mediating the constrictor effects of PE and U46619 was also seen in rat aorta (see Supplement Figure 2). At concentrations of U46619 below

30nmol/L there is a small ouabain-sensitive component (see Supplement Figure 2B). Interestingly, U46619 induces PLM phosphorylation (see Figure 1DF) which, because of its concomitant Na/K ATPase inhibition, is largely without effect. This PLM phosphorylation is likely to reflect our previous observation that interventions raising cytosolic Ca will increase PLM phosphorylation by activating Ca-dependent PKCs.[22] This may account for the small effect of ouabain on constriction in rat aorta at submaximal U46619 concentrations (i.e. below 30nmol/L) where the Na/K pump may still not be fully inhibited by U46619.

On the basis of these experiments, we conclude that $\alpha_1$ adrenoceptor stimulation activates signaling pathways leading to phosphorylation of phospholemman at Ser63 (presumably through a PKC dependent mechanism)[36] and Na/K ATPase activation. In WT tissue, this exerts a ouabain-sensitive 'relaxing' effect which limits the overall constrictor effect of PE. When PLM phosphorylation is prevented in PLM$^{3SA}$ tissue, or when the Na/K ATPase is inhibited ouabain (or U46619), this 'relaxing' effect is lost and constriction is potentiated.

***The PLM-dependent limitation of PE-induced constriction in WT aortae is partially blocked by L-NAME:***

Previous studies have shown that endothelially-derived factors modulate vascular responses to $\alpha_1$-adrenergic agonists via Na/K ATPase-dependent mechanisms.[4,15-18,37] Furthermore, we have previously demonstrated that NO exerts PLM-dependent modulation of Na/K pump in cardiac tissue.[38] We therefore hypothesized that at least part of the mechanism limiting PE-induced constriction in WT aortae may be NO-dependent. Supplement Figure 3A shows the enhanced response of PLM$^{3SA}$ tissues to PE (cf WT) (as in Figure 1A). Subsequent NOS inhibition with L-NAME (300µmol/L) increased the dose-dependent contractile response to PE in both genotypes but the relative potentiation was more profound in WT mice than PLM$^{3SA}$ (Supplement Figure 3). As a result of this potentiation, the difference in contractile responses between genotypes after NOS inhibition was substantially reduced but still statistically significant (see Supplement Figure 3B).

These data suggest that in WT aortae, the ouabain-sensitive mechanism limiting PE-induced constriction has two components – a NOS-dependent component (blocked by L-NAME) and a NOS-independent component (which persists in L-NAME).

***Aortic rings from WT mice are more sensitive to an NO donor***

To further investigate the role of PLM phosphorylation in NO-dependent regulation of the Na/K pump, in the next series of experiments we compared NO-dependent relaxation of aortic rings from both genotypes pre-constricted with a low concentration of U46619. The U46619 concentration used in these experiments was 40nmol/L as at this concentration there is still a ouabain-sensitive modulation of constriction (see Figure 2B) while, at higher concentrations, U46619 is reported to directly inhibit Na/K ATPase.[35] Initial tension after treatment with U46619 was the same across genotypes but spNONO-induced relaxation curves were left-shifted in aortic rings from WT mice, which reflects higher sensitivity of this genotype to NO (Supplement Figure 4A). Interestingly, pretreatment with ouabain (100µmol/L) profoundly limited the maximal relaxation response to NO and completely abolished the shift in the $K_m$ for spNONO between genotypes (Supplement Figure 4B). These results demonstrate that (i) PLM-dependent Na/K pump activation is one of the mechanisms involved in NO-dependent relaxation, (ii) Na pump-independent mechanisms are not different between genotypes and (iii) PLM phosphorylation facilitates NO-dependent relaxation of WT aortic rings. Further evidence for a role of PLM phosphorylation in NO-dependent relaxation was seen in immunoblot experiments. Treatment of isolated aortic segments from WT mice with spNONO (100 µmol/L) time-dependently increased PLM phosphorylation (Supplement Figure 4CDE).

Thus, these *in vitro* experiments show that PLM phosphorylation and consequent Na/K ATPase activation both limits the contractile response to PE and enhances NO-dependent relaxation. Our next aim was to test whether such *in vitro* observations made in aortic rings are also seen *in vivo* and to establish whether PLM phosphorylation therefore plays a role in regulating vascular tone and BP?

***Vascular constriction in response to PE is profoundly enhanced in PLM$^{3SA}$ mice in vivo***

We measured augmentation index (AI) in anesthetized mice as an index of aortic stiffness/tone *in vivo* (see Figure 5). AI was measured in WT and PLM$^{3SA}$ mice at baseline and during PE infusion (iv, 10-300 µg/kg). Baseline AI was not different between genotypes, but PE treatment induced a dose-dependent elevation of AI in both genotypes. Furthermore, the increase in AI in response to PE was significantly enhanced in PLM$^{3SA}$ compared to WT mice (Figure 2A).

PE-induced differences in AI could reflect differences in cardiac contractility, vascular stiffness, or both. To determine the contribution of any genotype-specific cardiac response to PE infusion, in separate experiments isolated hearts were treated with PE (Supplement Figure 5C). PE modestly increased contractility of both WT and PLM$^{3SA}$ hearts to an identical extent. We conclude that the greater PE-induced elevation of AI in PLM$^{3SA}$ mice reflects enhanced vascular contractility in this model.

In order to establish whether this enhanced vasocontractile response to PE in the PLM$^{3SA}$ mice is unique to aorta (or is a general feature of other vascular beds), blood flow in response to PE bolus injection was measured in hind-limb. Non-invasive laser Doppler hind-limb imaging in the two genotypes showed no differences in blood flow under baseline conditions (Figure 2B-D). However, acute bolus PE infusion (iv, 30-300µg/kg) induced a more profound reduction of blood flow in PLM$^{3SA}$ mice than in WT littermates (Figure 2B-D) at all PE concentrations. These data are consistent with the observation in Figure 2A of an enhanced constrictor response in aorta (AI) and augmented vascular contractility in PLM mutant mice in response to PE treatment *in vivo*.

***BP changes in PLM$^{3SA}$ mice: changes in baroreceptor gain and autonomic function***

Baseline BP was the same in conscious and anesthetized mice of both genotypes (Figure 3A, and Supplement Figure 6A). Given the profoundly enhanced constrictor response of PLM$^{3SA}$ mouse to PE both *in vitro* and *in vivo*, surprisingly, PE infusion (iv, 10-300 µg/kg) induced identical dose-dependent pressor responses in WT and PLM$^{3SA}$ mice (Figure 3A). This appears to be explained by differences between genotypes in their baroreceptor function. Figure 3B shows that the pressure-induced

bradycardia was far more profound in PLM$^{3SA}$ than WT mice. Baroreceptor sensitivity can be estimated by plotting the change in heart rate (RR interval) as a function of change in BP (Figure 3C). From this figure it is clear that the Baroreceptor Gain (BRG), as measured by the slope of this relationship, is almost 3 times higher in PLM$^{3SA}$ (1.42±0.11ms.mmHg$^{-1}$) than WT mice (0.5±0.07ms.mmHg$^{-1}$). This baroreceptor adaptation clearly allows the PLM$^{3SA}$ mouse to maintain normal BPs at rest, and over a range of pressor interventions, despite hyper-contractile vascular smooth muscle. Supplement Figure 6BC also shows that these changes in BRG allow the PLM$^{3SA}$ mice to maintain comparable changes in systolic BP in response to acute environmental stress by limiting heart rate changes. Further evidence for autonomic adaptation in these mice is shown in the heart rate variability (HRV) analysis in Figure 3D and autonomic balance (Supplement Figure 6D). PLM$^{3SA}$ mice show significantly reduced low to high frequency ratio (Figure 3D) consistent with a chronic adaptive reduced sympathetic dominance and altered autonomic balance (Supplement Figure 6D).

***Membrane potential measurements in mesenteric vessels: role of electrogenic Na/K pump activation?***

The high input impedance of vascular smooth muscle means that small changes in electrogenic Na/K pump activity can exert large effects on membrane potential ($E_m$) and hence constriction. We, therefore, hypothesized that the ouabain-sensitive differences in response to PE are mediated by changes in $E_m$. Figure 4A shows that at rest $E_m$ of WT mesenteric resistance vessels was -55±1.0mV. Of this, about -8mV can be attributed to the outward current carried by Na/K ATPase as ouabain, in WT vessels, reduces $E_m$ to -47±0.8mV. In the PLM$^{3SA}$ mouse, $E_m$ was considerably depolarized at rest (-50±0.5mV) and $E_m$ was no longer sensitive to ouabain - suggesting that, at rest, in the PLM$^{3SA}$ mouse, the contribution of the electrogenic Na/K ATPase to membrane potential is minimal. In WT mesentery (as in aorta – Figure 1DE) PLM was basally phosphorylated at Ser63 (Supplement Figure 7). However, unlike in aorta PE did not induce further PLM phosphorylation at either Ser63 or Ser68 (Supplement Figure 7). The ability of PE to induce PLM phosphorylation in aortic but not mesenteric vessels suggest that while the Na/K ATPase and PLM exert tonic effects on vascular tone and membrane potential,

the ability for this to be modulated by PE may vary in different vascular beds. The inability of PE to induce PLM phosphorylation also explains the similar constrictor responses to PE seen in WT and 3SA mesenteric vessels (Supplement Figure 8).

***Role of PLM de-phosphorylation in ageing-induced essential hypertension in mice***

Having demonstrated the importance of PLM phosphorylation in the regulation of vascular smooth muscle tone and BP in young mice, we investigated whether changes in PLM phosphorylation are associated with ageing-induced essential hypertension. Figure 5A shows the diurnal variation in mean arterial pressure (MAP) in young mice (14-16 weeks of age) and old hypertensive mice (57-60 weeks of age). In aortae from young mice, PLM was significantly basally phosphorylated at all 3 serine residues (Figure 5DE). However, in old hypertensive mice PLM was very substantially hypo-phosphorylated and over-expressed (Figure 5DE). There were no significant differences in $\alpha$1 or $\alpha$2 Na/K ATPase subunit expression with age (Supplement Figure 9A) but the increase in PLM expression resulted in a significant relative excess of unphosphorylated PLM in old mice (Supplement Figure 9B).

BP in young and old wild-type and $PLM^{3SA}$ mice is shown in Figure 5BC. While wild-type mice show substantial ageing-induced hypertension both during the day and night, $PLM^{3SA}$ mice are completely protected against hypertension at rest (day) and very substantially protected against hypertension during activity (night). In old WT mice average MAP at rest (day) was +13mmHg higher than in young mice, this hypertension was completely prevented in $PLM^{3SA}$ mice. During activity (night) old WT mice showed a substantial ageing-induced hypertension (+18mmHg) which was substantially abrogated in the $PLM^{3SA}$ genotype (+10mmHg). The heart rate variability differences seen in young mice (Figure 3D) were still present in aged mice (Supplement Figure 10). Not only were $PLM^{3SA}$ mice protected against ageing-induced hypertension but they also were protected against ageing-induced cardiac dysfunction (Figure 5F and Supplement Figure 11).

***A homologous varient in a human population influences BP***

We identified a single nucleotide polymorphism (SNP) in PLM that generates an R70C amino acid substitution in PLM. We hypothesized that R70C is unlikely to influence phosphorylation at PLM Ser68 by PKA (consensus motif RRXS/T). PKC isoforms generally require positive charges in positions +3, +2, -2 and -3 (consensus RRXS/TXRR),[39] so we hypothesised that phosphorylation by PKC at Ser68 (but not Ser63 and Thr69) may be perturbed (Figure 6A). We also investigated the possibility that mutation R70C destroys the proposed endoplasmic reticulum retention motif (RRR) in PLM.[40] In HEK cells, the R70C PLM mutation is not phosphorylated at Ser68 by PKC (Figure 6BC), and displays enhanced cell surface localisation and palmitoylation (Supplement Figure S12) while phosphorylation at the more remote Ser63 site by PKC was unaffected by the R70C mutation (Figure 6D).

To assess the impact of this SNP, we analysed data from UKBB including a total of 357,151 unrelated individuals of European ancestry, with 159,204 males and 197,947 females (Supplementary Table 1). In total, 7,114 individuals carried at least one copy of the rare T allele of the SNP rs61753924, with a Minor Allele Frequency (MAF) of 1% and no evidence of departure from Hardy Weinberg Equilibirum (p=0.33). At recruitment, mean age was 56.4yrs, mean systolic BP (SBP) was 140.5mmHg and mean diastolic BP (DBP) was 84.1mmHg, with 50.2% of indivudals being hypertensive (SBP ≥ 140mmHg or DBP ≥ 90mmHg) and 17.2% reported to be taking BP lowering medication.

Within UKBB this SNP is associated with BP in a sex-and age-dependent manner (Figure 7). The rare T allele is significantly associated with increasing levels of both SBP (0.77mmHg, 95% CI 0.14-1.4, p=0.017) and DBP (0.4mmHg, 95% CI 0.02-0.79, p=0.04) in males. However, there is no significant association with BP in either the overall sample (p=0.16 for SBP; p=0.11 for DBP) or within females (p=0.85 for SBP; p=0.75 for DBP). Hence this suggests a sex-specific association. Focusing on results within males, Figure 7A illustrates an age-dependent effect of this SNP, with the association being significant only in the age-stratified analyses for males aged 45-50 yrs and 50-55 yrs old. Indeed an initial age-stratified analysis comparing males aged ≤55 yrs old vs males aged >55 years old shows that the rare T allele is significantly associated with increasing levels of both SBP (1.2mmHg, 95% CI 0.30-2.11, $p=8.89\times10^{-3}$) and DBP (0.79mmHg, 95% CI 0.18-1.4, p=0.01) in males under 55yrs old, but is not

significantly associated with BP in males over 55yrs old (p=0.28 for SBP; p=0.48 for DBP). We therefore conclude that the SNP has strongest effect in middle-aged men, with the rare T allele significantly increasing both SBP (1.7mmHg, 95% CI 0.59-2.8, $p=2.62\times10^{-3}$) and DBP (1.02mmHg, 95% CI 0.29-1.74, $p=5.96\times10^{-3}$) in males aged 45-55yrs old (Figure 7E).

In a similar analysis in the GoDARTS cohort (n=7,784) the proportion of individuals carrying the T allele was slightly higher at 3%, compared to UKBB although in Hardy-Weinberg equilibrium (p=0.7). We replicated the findings from UKBB with the T allele showing significant association with SBP increased by 4.5mmHg, 95%CI 0.72-8.27 (P=0.02) only in middle-aged males aged of 40-65 years.

## DISCUSSION

These studies demonstrate the importance of phospholemman (PLM)-mediated regulation of vascular smooth muscle Na/K ATPase activity, membrane potential, tone and BP. PLM phosphorylation stimulates Na/K ATPase which limits agonist-induced vascular smooth muscle depolarisation and hence limits constriction (see Figure 8). The presence of a SNP that abolishes PLM phosphorylation at Ser68 by PKC is significantly associated with increased BP in middle-aged men. Mutation of the phosphoregulatory sites on PLM in mice blocks this pathway and renders vascular smooth muscle hyper-responsive to phenylephrine. These $PLM^{3SA}$ mice show a chronic adaptation of their baroreceptor sensitivity which can maintain normal BP at rest in response to exogenous phenylephrine (or environmental stress) despite a profoundly enhanced peripheral vasoconstriction. The autonomic adaptations in these mice can prevent chronic essential hypertension induced by ageing. In aged wild-type mice the combination of increased PLM expression and profound PLM hypo-phosphorylation is associated with hypertension which was largely abrogated by autonomic adaptations in $PLM^{3SA}$ mice. This raises the possibility that PLM and the prevention of its dephosphorylation and Na/K ATPase inhibition may provide a novel therapeutic target in ageing-induced essential hypertension.

***Mechanism of PLM-mediated modulation of vasoconstriction:***

A number of studies have shown that Na/K ATPase modulates vascular tone independent of changes in intracellular Na.[3,41,42] We therefore propose that activation of vascular smooth muscle Na/K ATPase, and the PLM-mediated ouabain-sensitive relaxing effect, involves increased electrogenic Na/K pump current which hyperpolarizes the cell membrane or limits agonist-induced depolarisation. Preventing PLM phosphorylation in $PLM^{3SA}$ mice profoundly depolarizes resting membrane potential in mesenteric arteries from -55±2mV (WT) to -50±1mV ($PLM^{3SA}$). The steep current-voltage relationship of the L-type Ca channel window-current means that such a change in membrane potential can significantly increase steady-state Ca influx and hence increase vascular tone in 3SA vessels. In WT vessels, this ouabain-sensitive 5mV hyperpolarisation persisted across the entire range of depolarisations induced by PE. In these mesenteric vessels this hyperpolarisation was not further enhanced by a concentration-dependent PE-induced Na/K pump activation as PLM phosphorylation was not further increased by PE treatment (unlike in aorta).

***Differences in the role of PLM in U46619 and PE-induced constriction:***

Our data suggests that the constrictive effects of PE are limited by two factors – PLM phosphorylation activating the Na/K ATPase and (ii) endothelially derived NO release. Neither of these occur in U46619 constriction as the Na/K ATPase is already inhibited by U46619 and U46619 has been reported to inhibit rather than promote NO release from the endothelium.[43,44]

***Role of nitric oxide (NO) in PLM modulation of relaxation:***

Supplement Figure 4 also shows that nitric oxide (NO), as in cardiac muscle,[38] can also induce PLM phosphorylation. Hence a component of classical NO-mediated relaxation is likely to involve the activation of Na/K ATPase via this mechanism. As PE also stimulates the generation of NO,[37] it can therefore limit constriction through direct PKC-mediated phosphorylation of PLM and also via more classical NO-dependent downstream pathways which are PLM-independent.

NOS inhibition significantly augmented dose-dependent constriction to PE in both genotypes (Supplement Figure 3) demonstrating the presence of a PLM-independent effect of PE that antagonizes constriction. However, potentiation of constriction by L-NAME was most profound in the WT aortic segments (Supplement Figure 3) suggesting that PLM phosphorylation plays a role in NO-dependent modulation of PE-induced constriction. Interestingly, even in the presence of L-NAME, aortic constriction to PE was higher in PLM$^{3SA}$ mice than in WT (Supplement Figure 3) demonstrating that PE activates a complex array of NO-dependent/independent and PLM-dependent/independent mechanisms that antagonize PE-induced constriction.

The assumption is that the 3SA mutation mediates its effects by influencing Na/K ATPase activity in vascular smooth muscle. However, the 3SA is a global knock-in under the control of the endogenous PLM promotor. However, FXYD proteins are ubiquitous and it is likely that vascular endothelium will express its own FXYD protein which has yet to be identified. It seems unlikely that this will be FXYD1 (PLM) as all reports suggest that this is muscle-specific. We have failed to detect PLM in endothelial cell (HUVEC) cultures (data not shown). None of the other members of the FXYD family contain the cytoplasmic tail with the regulatory phosphorylation site and so they could not be candidates for modulation by phosphorylation. Should aortic vascular endothelium express FXYD1 (PLM) then this could affect the Na gradient in endothelial cells and hence intracellular Ca and NOS-induced NO release.

***In vivo adaptations and control of blood pressure***

The role of PLM phosphorylation in the regulation of vascular tone was also confirmed *in vivo*. Augmentation Index (AI) is influenced by the velocity and amplitude of the aortic pressure wave reflection and is proportional to arterial stiffness.[45-48] In PLM$^{3SA}$ mice, AI was similar to that in WT at baseline but the response to acute administration of PE was profoundly amplified in PLM$^{3SA}$ mice (Figure 2A). In addition, hind limb resistance vessels also show enhanced constriction in response to bolus infusion of PE in PLM$^{3SA}$ mice (Figure 2BCD).

Surprising, despite this profoundly enhanced vascular responsiveness, *in vivo* BP was not different between the genotypes even during PE infusion (Figure 3A). This lack of enhanced pressor response to PE despite a profound increase in both central and peripheral vasoconstriction is explained by an enhanced reflex bradycardia (Figure 3B). This is best demonstrated by the change in baroreflex gain in Figure 3C. The PLM$^{3SA}$ mouse has a baroreflex sensitivity almost 3 times that of the wild-type mouse (1.42±0.11 vs 0.5±0.07ms.mmHg$^{-1}$). There also appears to be an autonomic compensation under baseline conditions that is reflected in reduced LF/HF Ratio (sympathetic dominance) of Heart Rate Variability (Figure 3D) measured in conscious telemetered mice. Pharmacological inhibition of sympathetic and parasympathetic activity revealed substantially lower activity in both limbs of the autonomic nervous system and lower intrinsic heart rate in PLM$^{3SA}$ mice (Supplement Figure 6D). New environment stress induced similar elevation of BP in both genotypes but again the chronotropic response was limited in PLM$^{3SA}$ mice (Supplement Figure 6BC). Thus, different autonomic control of heart rate, lower intrinsic pacemaker activity and higher baroreflex sensitivity appear to underlie adaptive mechanisms allowing PLM$^{3SA}$ mice to maintain normal arterial BP despite the absence of PLM-dependent modulation of vascular function.

***Phospholemman phosphorylation and ageing-induced essential hypertension***

Ageing in WT mice is associated with essential hypertension (Figure 5AC) and a gradual increase in arterial stiffness.[49] There is a profound age-related reduction of PLM phosphorylation at all PLM phosphorylation sites accompanied by increased PLM expression in aortic tissue (Figure 5DE). This combination of increased PLM expression and profound dephosphorylation will inhibit Na/K ATPase. A reduction in Na/K ATPase activty may not only affect vascular tone, as decsribed in this study, but may also lead to chronic vascular remodelling.[7] PLM$^{3SA}$ mice were very significantly protected against this ageing-induced hypertension and HRV analysis suggests that these old PLM$^{3SA}$ mice maintain their adaptive changes in autonomic function in a way that protects them against ageing-induced hypertension (Supplement Figure 10).

Our previous studies demonstrated that PLM$^{3SA}$ mice are more prone to cardiac hypertrophy and dysfunction in a banding model of heart failure.[29] However, it is interesting to note that aged PLM$^{3SA}$ mice in this study show no cardiac hypertrophy, and reduced contractile dysfunction compared to aged WT littermates (Figure 5F and Supplement Figure 11). This suggests that the reduction in the trigger for hypertrophy (ie essential hypertension) in ageing PLM$^{3SA}$ mice can compensate for the previously shown vulnerability of these mice to pressure overload.

While it is tempting to suggest that the down-regulation of Na/K pump function in WT mice is causally associated with ageing-induced hypertension, and the protection afforded in the PLM$^{3SA}$ mouse suggests a causal role for PLM phosphorylation, it is also possible that the chronic down-regulation of sympathetic outflow in the PLM$^{3SA}$ mouse is akin to a life-time of beta blockade and the changes in WT PLM are merely co-incidental.

***Therapeutic potential of targeting the PLM/NKA regulatory axis in hypertension***

In humans, the R70C PLM mutation (rs61753924) has a significant impact on BP profiles. At middle age possession of the T allele is associated with both increased SBP and DBP levels. In particular, middle-aged male carriers have 1.7mmHg higher mean SBP. Within the general population, an effect of this size for a single SNP alone is substantial. The results of BP genetic association studies, considering the effect of all common variants (MAF≥1%) associated with BP, show that the maximum effect size for a single common variant is ~1.2mmHg for SBP, with the average effect size across all SBP-associated SNPs being ~0.3 mmHg.[50] Furthermore, even the genetic risk score combining together the effects of all 901 independent BP-associated SNPs reported in 2018 shows an aggregated risk of only ~10mmHg difference in mean SBP when comparing individuals in the top vs bottom 20% of the genetic risk distribution. Of course, there is an increasing trend between the effect size of associated SNPs and their MAF, with only rare variants expected to have larger effects, and indeed this is a low-frequency SNP with MAF=1%, however even in recent BP genetic studies investigating rare variants, very few individual SNPs have still been identified with an effect size for SBP of >2mmHg.[51] We note

however, that due to the rare frequency of this SNP, and considering the highly polygenic nature of BP within the general human population, we do not claim any strong clinical impact for this SNP alone. Instead, it is only really genetic mutations for monogenic hypertension syndromes which are expected to have larger, clinically meaningful effect sizes for single mutation variants.[50,51] Therefore we suggest that the large effect size of this SNP for increasing BP levels within middle-aged men demonstrates the importance of the PLM/Na/K ATPase regulatory pathway in the overall control of BP. PLM-mediated Na/K ATPase activation may offer therapeutic potential for poorly-managed essential hypertension.

**ACKNOWLEDGEMENTS**

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**DISCLOSURES**

None

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**FIGURES**

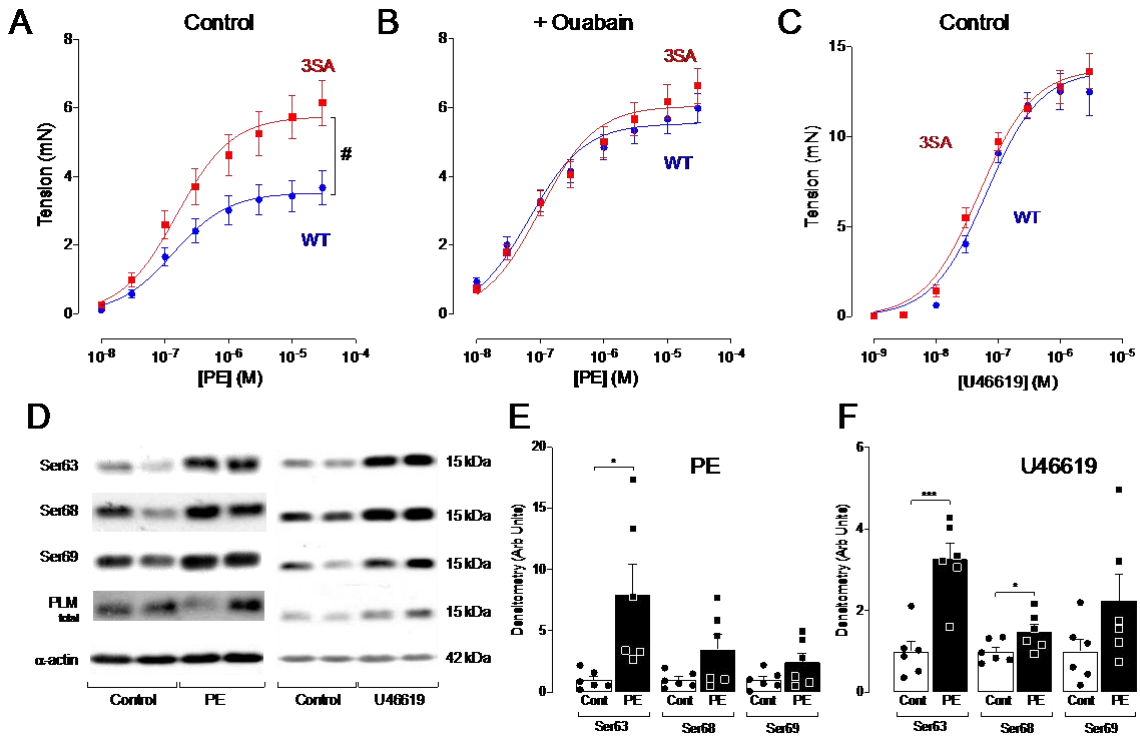


***Figure 1:*** **Phospholemman (PLM) phosphorylation limits phenylephrine-induced vasoconstriction in wild-type (WT) isolated mouse aortic rings in a ouabain-sensitive manner. Mutation of PLM to prevent phosphorylation (3SA) enhances constriction. Constriction in response to the thromboxane $A_2$ mimetic U46619, which also increases PLM phosphorylation, was unaffected by genotype. A:** Mutation of PLM phosphorylation sites to prevent phosphorylation (3SA) markedly enhanced vasoconstriction (n=6 (WT) and n=5 (3SA), #P<0.001 2-way ANOVA for $V_{max}$ comparison). **B:** The limitation of vasoconstriction in wild-type (WT) aortae was completely blocked by pretreatment with ouabain (300µmol/L) (n=5/group). **C:** Contractile response to the thromboxane A2 mimetic U46619 was unaffected by genotype (n=6 (WT) and n=7 (3SA)). **D, E and F:** In WT aortae both phenylephrine (10µmol/L) and U46619 (1 µmol/L) markedly increased PLM phosphorylation (n=6/group, *P<0.05, ***P<0.001 unpaired t-test).

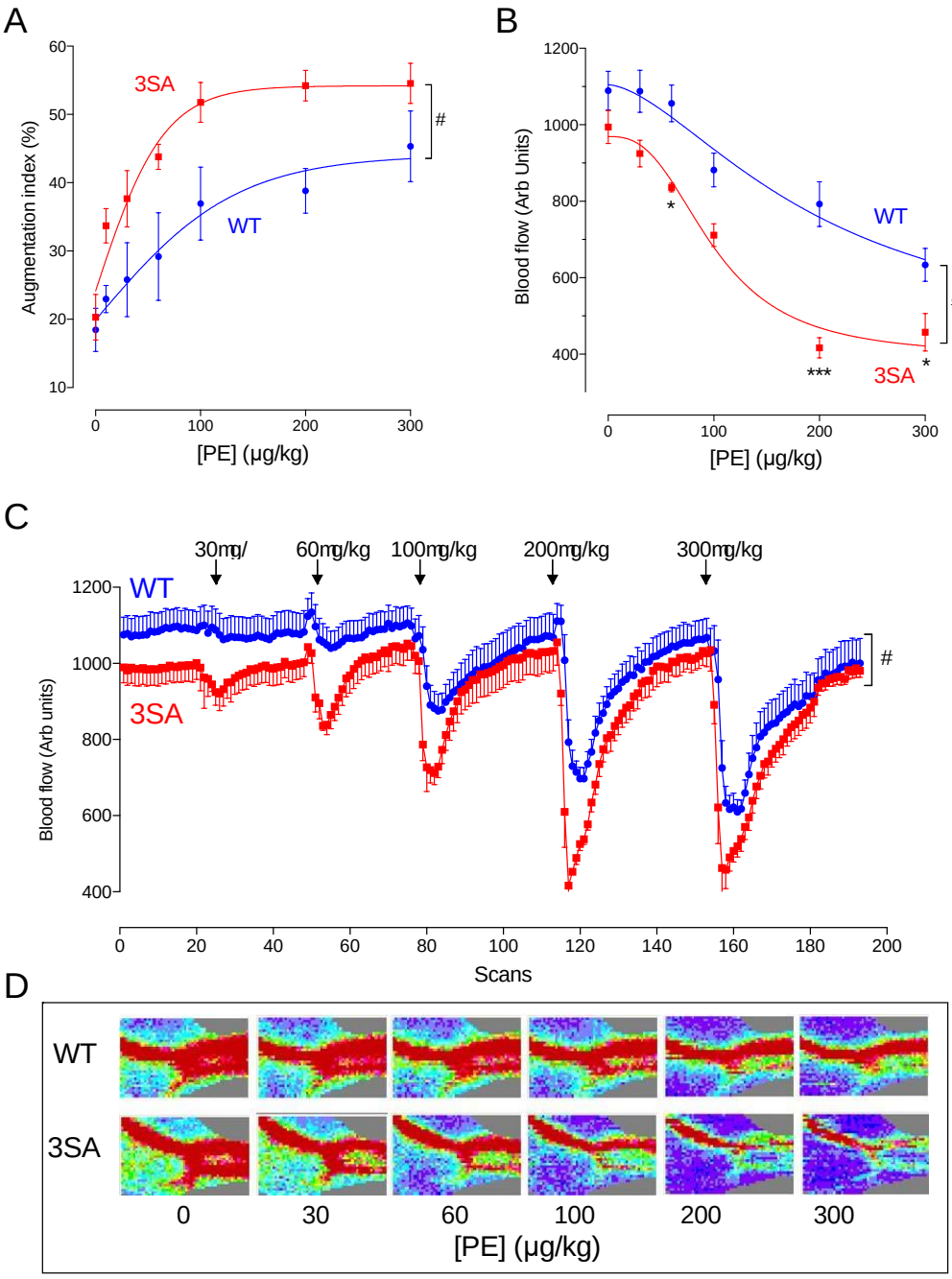


***Figure 2: In vivo* measurements of aortic stiffness as assessed by augmentation index, and peripheral blood flow measured in anaethetized wild-type and PLM$^{3SA}$ (3SA) mice in response to phenylephrine.** Phenylephrine (PE) was infused as a bolus injection via a left jugular vein catheter at the doses indicated. **A:** Aortic pressure measurements were made via a pressure tipped catheter (1.2F, Transonic) introduced into the ascending thoracic aorta via the right carotid artery. Augmentation Index was measured as the percentage increase in aortic pressure from the first inflection in the aortic pressure trace to the subsequent peak pressure (n=5 for both groups). **B-D**: In separate experiments, peripheral blood flow was measured in the hind-limb by laser Doppler in response to PE bolus infusion (n=5 (3SA) and n=6 (WT)). In Panels A, B and C comparisons were made using 2-way ANOVA with Bonferroni correction and #P<0.001 for effect of both genotype and PE concentration and specifically *P<0.05, ***P<0.001.

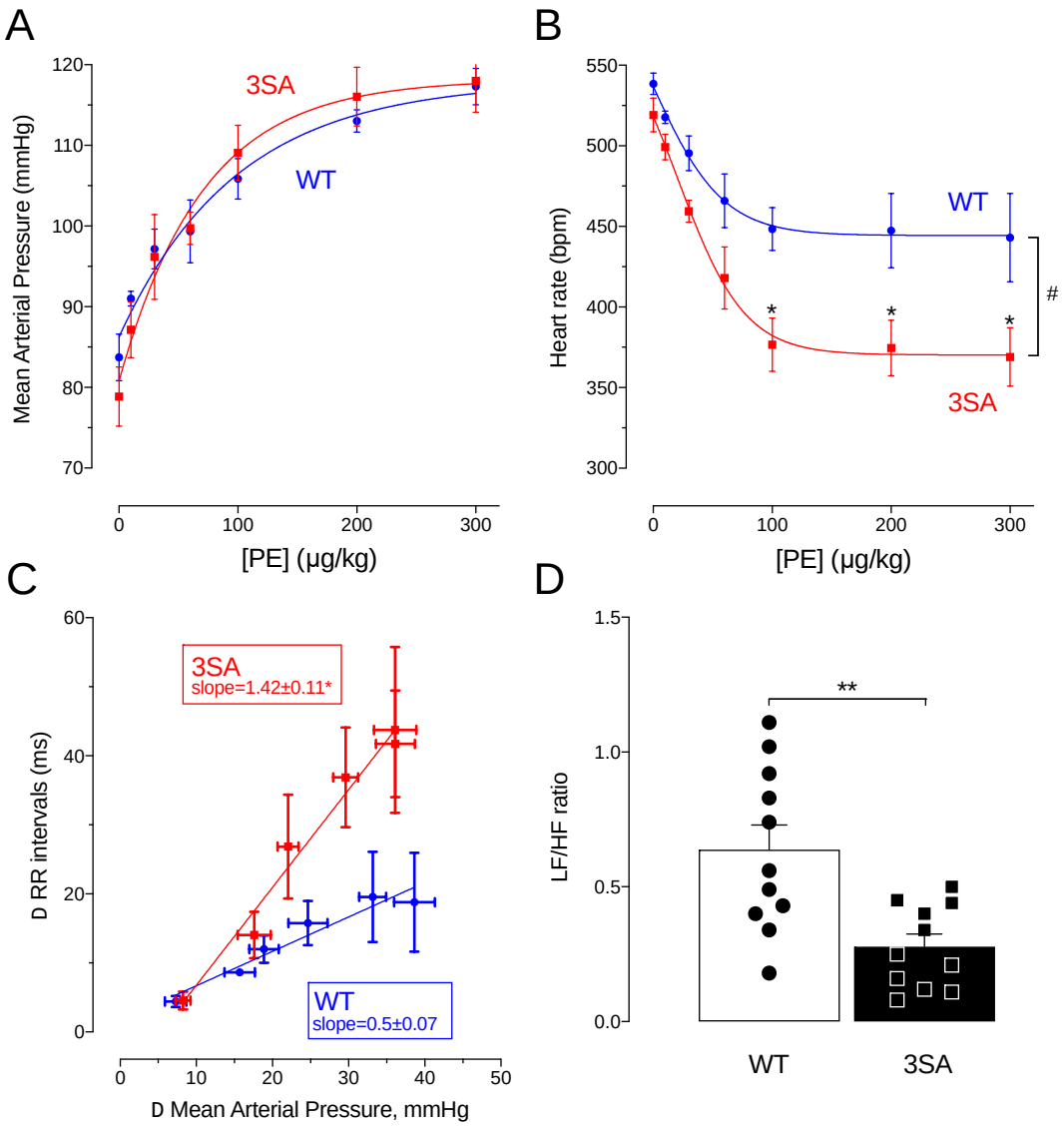


***Figure 3: In vivo* measurements of Mean Arterial Pressure (MAP), heart rate, baroreceptor reflex gain and autonomic function in anaethetized wild-type and PLM$^{3SA}$ (3SA) mice in response to phenylephrine.** Phenylephrine (PE) was infused as a bolus injection via a jugular vein catheter at the doses indicated. **A:** Mean Arterial Pressure measurements were made as described in Figure 2 and heart rate (**B**) was a calculated from the pressure trace. Baroreceptor reflex gain (BRG) was estimated by plotting the change in beat-beat interval (ΔRR) as a function of change in MAP (ΔMAP) in response PE infusion (**C**) with BRG estimated as the slope of this relationship (*P<0.05). The Low/High frequency component (Sympathetic dominance) of Heart Rate Variability (HRV) measured in conscious telemetered mice under control conditions is shown in Panel **D**. (Panels A-C, n= 5/group) and Panel D, n=11/group. In Panel B comparison were made using 2-way ANOVA with Bonferroni correction and #P<0.001 for effect of both genotype and PE concentration and specifically *P<0.05. Difference in Panel D was tested using an un-paired t-test **P<0.005.

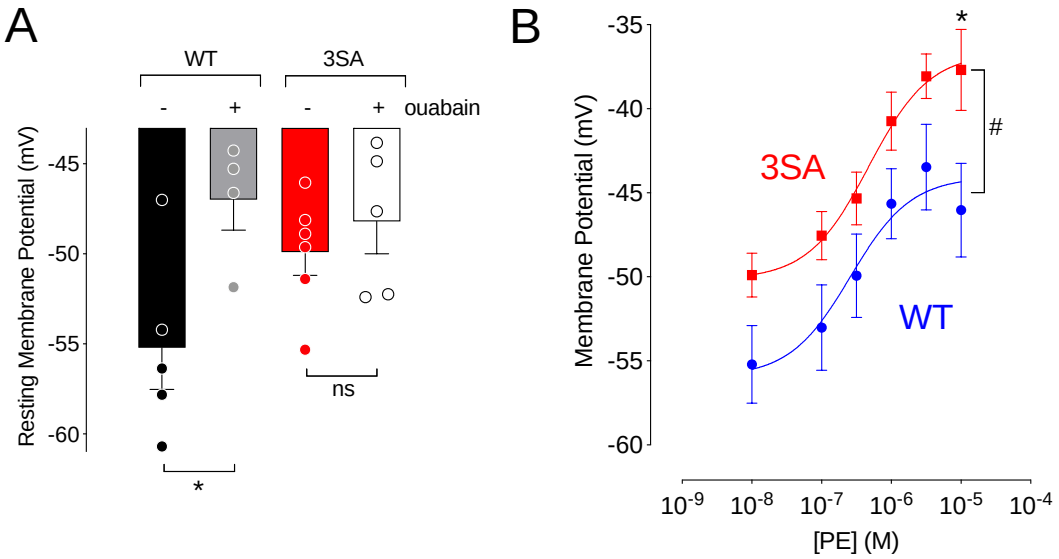


***Figure 4:*** **Effect of phenylephrine (PE) on membrane potential measured in mesenteric vessels isolated from wild-type (WT) and PLM$^{3SA}$ (3SA) mice: A:** Effect of ouabain (300µmoles/L) on basal membrane potential measured in WT and 3SA mesentery. Resting membrane potential was more hyperpolarized in WT than 3SA vessels – an effect which was lost when vessels were exposed to ouabain. Comparisons were made using 1-way ANOVA followed by an un-paired t-test (*P<0.05). **B:** Phenylephrine (PE) induces an equivalent dose-dependent net depolarization in both 3SA and WT vessels with the WT vessels starting approximately 5mV more hyperpolarized. Comparisons were made using 2-way ANOVA with Bonferroni correction and #P<0.0001 for effect of both genotype and PE concentration and specifically *P<0.05 (n=4 (WT) and n=6 (3SA)).

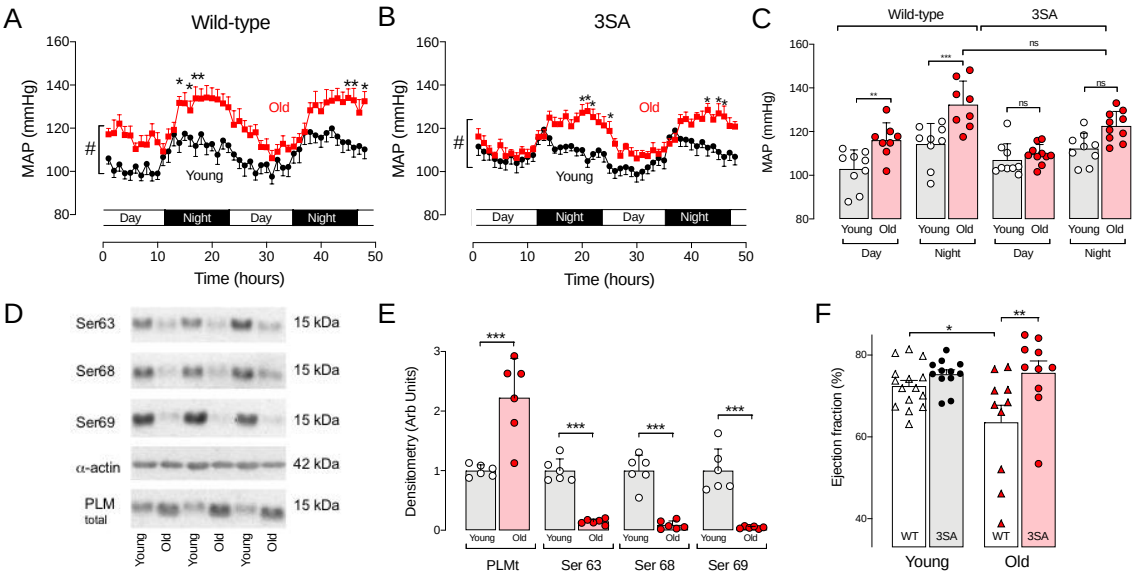


***Figure 5:*** **Ageing-induced essential hypertension was associated with significant PLM hypo-phosphorylation in wild-type mice. PLM$^{3SA}$ mice were substantially protected against both this ageing-induced hypertension and the associated cardiac hypertrophy and contractile dysfunction**. **A:** Ageing-induced essential hypertension measured over 48 hours in telemetered young (14-16 weeks) and old (57-60 weeks) wild-type mice. **B:** In 3SA mice, the diurnal fluctuation of BP was similar to that in young WT mice but nocternal hypertension was significantly attenuated in 3SA mice. **C:** Average Mean Arterial Pressures (MAP) recorded in young and old mice during day and night-time by genotype. **D and E**: In WT mice PLM was substantially hypo-phosphorylated at serines 63, 68 and 69 in aortic tissue from old mice (n=6/group, *P<0.05). **F:** PLM$^{3SA}$ mice were also protected against ageing-induced cardiac contractile dysfunction (ejection fraction) (See also Supplement Figure 11). In Panels A and B comparison were made using 2-way ANOVA with Bonferroni correction and #P<0.001 for effect of both age and time and specifically *P<0.05. In Panels C and F comparison were made using 1-way ANOVA with Bonferroni correction and in Panel E unpaired t-test was used for comparison. n=10 (adult, WT), n=9 (old, WT), n=11 (adult, PLM$^{3SA}$), n=11 (old, PLM$^{3SA}$), *P<0.05. **P<0.01. ***P<0.001.

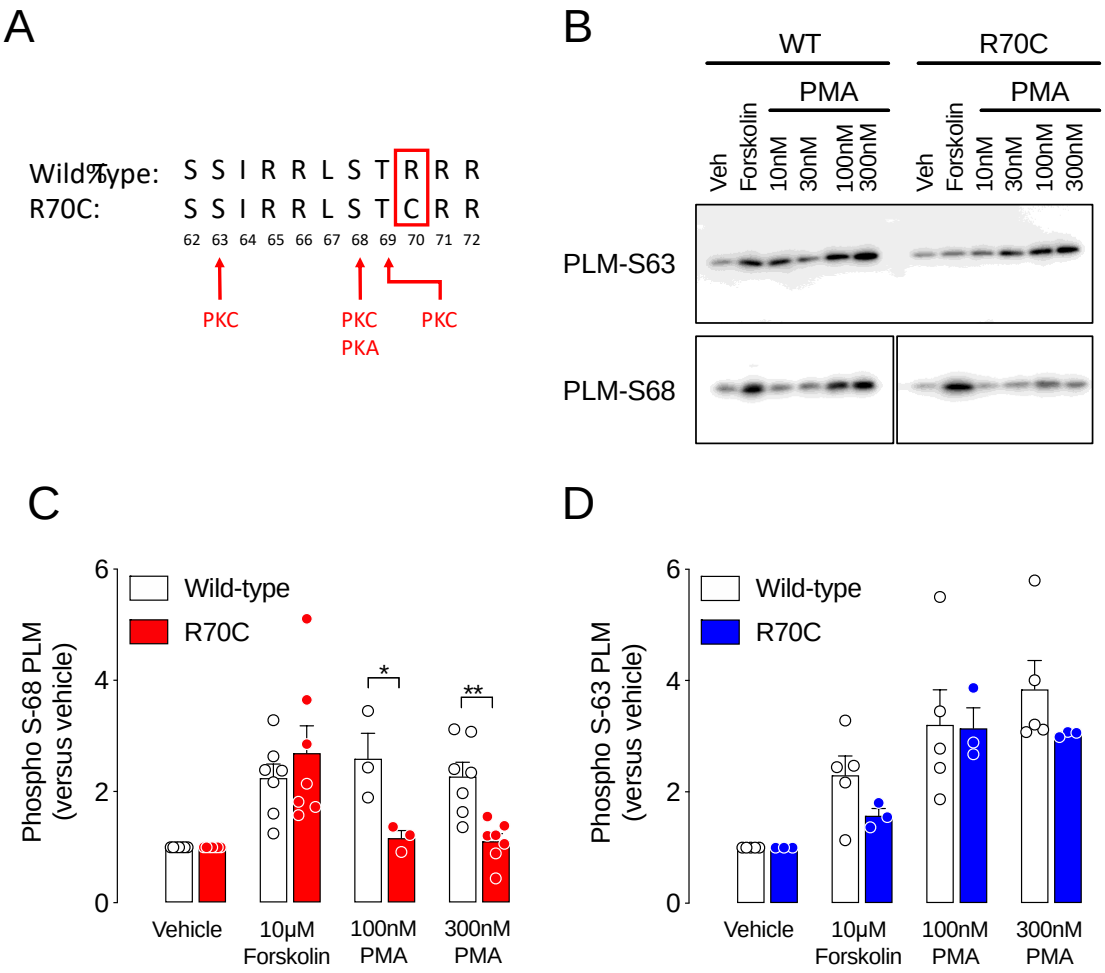


***Figure 6:*** **The R70C mutation limits PKC but not PKA phosphorylation of PLM at Ser68 but does not affect phosphorylation at the more remote Ser63. A:** Position of the R70C mutation in human PLM. The PKC and PKA phosphorylation sites at Ser63, Ser68 and T69 are indicated. **B:** Phosphospecific immunoblots after activation of PKA or PKC in transfected WT and R70C HEK cells. **C:** and **D:** quantified PLM Ser68 and Ser63 phosphorylation respectively. In Panels C and D unpaired t-test was used for comparison (C: n=7/grp and D: n=3-5). *P<0.05; **P<0.005. All other comparisons between WT and R70C not significant.

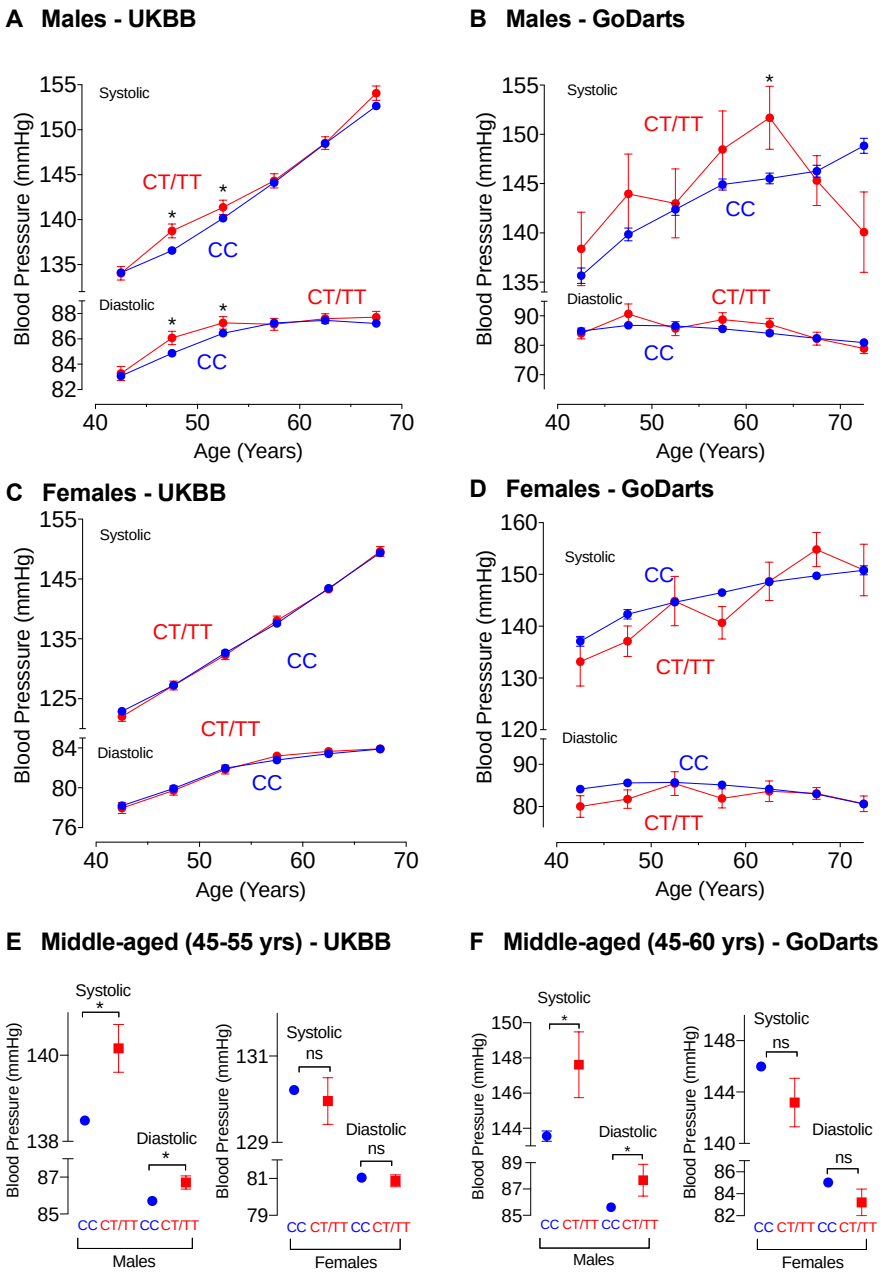


***Figure 7: The R70C phospholemman mutation (rs61753924) is associated with an age- and sex-dependent effect on BP in two human cohorts - UK BioBank and GoDARTS.*** CC = comparitor (control) and CT and TT represent heterozygote and homozygote genotypes. Mean systolic and diastolic BPs, within 5-year age intervals, are shown in both cohorts. The T-allele was associated with a significant increase in both systolic and diastolic BP in males (**A** and **B**) but not females (**C** and **D**), and especially associated within middle-aged males (**E** and **F**). Plots A-D plot the mean BP estimates within each age-group and tests for significant differences between consecutive age groups, according to non-overlapping 95% Confidence Intervals. Plots E-F show the mean BP estimates within middle-aged subjects corresponding to results from the age-stratified linear regression analyses, testing for a significant difference in mean BP between CC vs CT/TT genotype groups. Sample sizes: UKBB: n=159,204 males (A), 197,947 females (C), 44,995 middle-aged (45-55yrs) males and 58,194 middle-aged females (E). GoDarts: n=4,460 males (B), 3,324 females (D), 1,786 middle-aged (45-60yrs) males and 1,187 middle-aged females (F). *P<0.05.

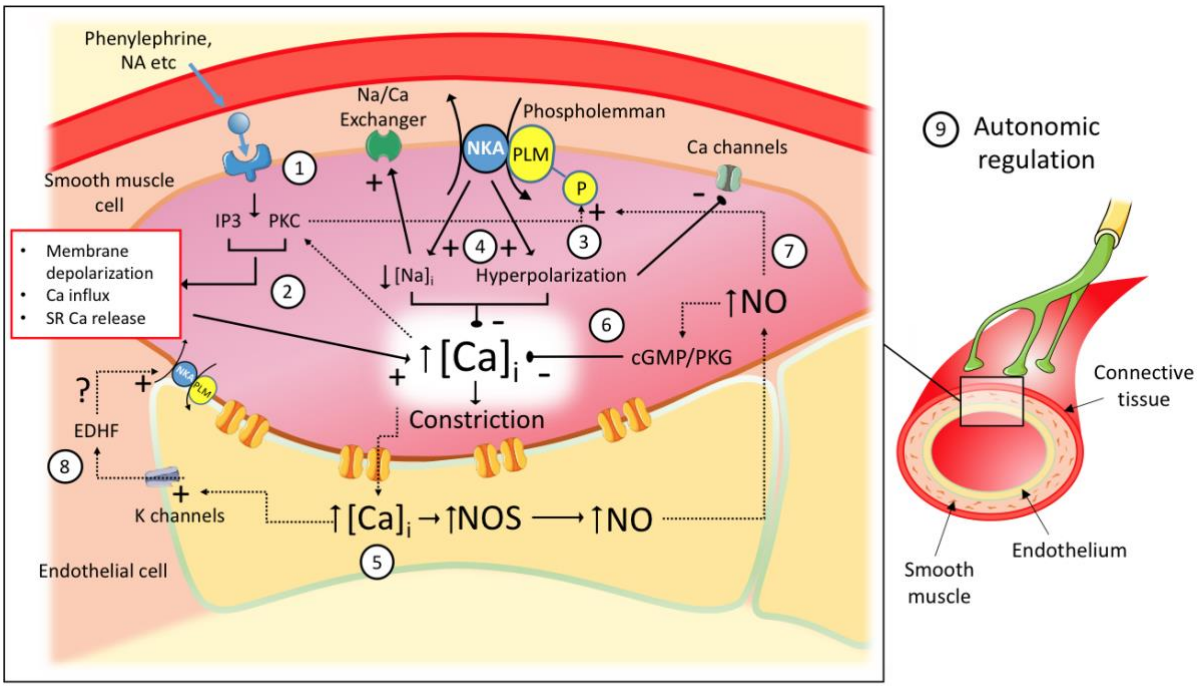


***Figure 8:*** **Proposed mechanisms by which phospholemman (PLM) phosphorylation and Na/K ATPase activation modulates vascular contractility: (1):** Activation of cell surface receptors ie by phenylephrine or noradrenaline (NA) activates IP3 and protein kinase C (PKC). **(2):** This elevates cytosolic Ca through a combination of membrane depolarization and enhanced Ca entry (principally via voltage-gated channels) and cyclic sarcoplasmic reticulum (SR) Ca release leading to constriction. **(3):** PKC, whose activation is enhanced by elevation of cytosolic Ca, phosphorylates phospholemman (at either Ser63 or both Ser63 and Ser68). (**4**): Phosphorylated PLM dis-inhibits the Na/K ATPase (NKA) which generates an outward current limiting the depolarization of the cell membrane and hence limiting Ca influx through voltage-gated channels and/or lowers cytoplasmic Na facilitating Ca efflux via Na/Ca exchange. This limits the rise in Ca in the cytosol and hence limits constriction. **(5):** The receptor-mediated rise in cytosolic Ca also passes to adjacent endothelial cells where it activates both nitric oxide synthase (NOS) and the release of EDHF. Nitric oxide (NO) diffuses to smooth muscle cells where it limits constriction by two pathways (i) via the canonical cGMP/PKG pathway **(6)** and (ii) via activation of a PLM/NKA-dependent pathway **(7).** EDHF release from endothelial cells may also limit depolarization possibly via activation of ouabain-sensitive NKA **(8)**. Basal phosphorylation of PLM influences vascular tone via these mechanisms and, in the PLM$^{3SA}$ mouse, adaptive changes in baroreceptor gain and autonomic control maintain baseline resting BP despite enhanced vascular reactivity **(9).** In aged mice, and in humans expressing the R70C mutation, defects in this PLM/NKA mechanism contribute to hypertension.