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Plasma membrane calcium ATPases (PMCAs) as potential targets for the treatment of essential hypertension

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ABSTRACT

The incidence of hypertension, the major modifiable risk factor for cardiovascular disease, is increasing. Thus, there is a pressing need for the development of new and more effective strategies to prevent and treat hypertension. Development of these relies on a continued evolution of our understanding of the mechanisms which control blood pressure (BP).

Resistance arteries are important in the regulation of total peripheral resistance and BP; changes in their structure and function are strongly associated with hypertension. Anti-hypertensives which both reduce BP and reverse changes in resistance arterial structure reduce cardiovascular risk more than therapies which reduce BP alone. Hence, identification of novel potential vascular targets which modify BP is important.

Hypertension is a multifactorial disorder which may include a genetic component. Genome wide association studies have identified *ATP2B1*, encoding the calcium pump plasma membrane calcium ATPase 1 (PMCA1), as having a strong association with BP and hypertension. Knockdown or reduced PMCA1 expression in mice has confirmed a physiological role for PMCA1 in BP and resistance arterial regulation. Altered expression or inhibition of PMCA4 has also been shown to modulate these parameters. The mechanisms whereby PMCA1 and 4 can modulate vascular function remain to be fully elucidated but may involve regulation of intracellular calcium homeostasis and/or comprise a structural role. However, clear physiological links between PMCA and BP, coupled with experimental studies directly linking PMCA1 and 4 to changes in BP and arterial function, suggest that they may be important targets for the development of new pharmacological modulators of BP.

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1. Introduction

Hypertension is a major public health issue and is responsible for considerable morbidity and mortality. As such, there is a continued need for the identification of new targets for more effective treatments for high blood pressure (BP). Changes in the structure and function of resistance arteries play a key role in the development of hypertension (Heagerty et al., 2010; Rizzoni & Agabiti-Rosei, 2012).

Abbreviations: AngII, angiotensin II; *Atp2b1*, gene for PMCA1 protein; BP, blood pressure; [Ca^{2+}], intracellular free calcium concentration; GWAS, genome-wide association study; NCX, Na^{+}/Ca^{2+} exchanger; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide; NO, nitric oxide; PMCA, plasma membrane calcium ATPase; SERCA, sarcoplasmic reticulum calcium ATPase; VSMC, vascular smooth muscle cell; VSMCs, vascular smooth muscle cells.

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Plasma membrane calcium ATPases (PMCAs) are ATP driven calmodulin-dependent pumps. PMCAs extrude Ca^{2+} ions from the cell and may thus contribute to the regulation of global, and sub-cellular, concentrations of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) (Karaki et al., 1997; Carafoli et al., 2001; Cartwright et al., 2011). However, it is now evident that PMCAs also have important roles as scaffold proteins whereby they may play a key role in signal transduction (Schuh et al., 2001; Williams et al., 2006; Cartwright et al., 2007; Strehler, 2015). Mammalian PMCAs belong to a multigene family of four different genes (ATP2B1–4 in humans) which encode respectively for PMCA proteins 1–4. The protein expression of PMCA2 and 3 is limited, principally to neuronal-type cells, however, PMCA1 and 4 are expressed throughout the body (Carafoli, 1991; Keeton et al., 1993; Street et al., 1998; Okunade et al., 2004).

In this review we focus on essential (primary) hypertension and provide an overview of current knowledge relating to the regulatory effects of PMCAs on the resistance vasculature and effects on BP. Based on this we present an argument to support PMCA-mediated pathways as promising new targets for the treatment of hypertension. Whilst we recognise the importance of cardiac function in relation to BP control such a broad topic is beyond the scope of this review. PMCAs provide only a minor contribution to expulsion of total Ca^{2+} from cardiomyocytes (Bers, 2000). However, ongoing studies seek to clearly define the role of PMCAs in other cardiac cell types and under pathological conditions, and we direct interested readers to some of the publications in this area (Oceandy et al., 2007; Mackiewicz et al., 2009; Wu et al., 2009; Chen et al., 2010; Cartwright et al., 2011).

2. Hypertension

2.1. A global clinical issue

Hypertension is the major modifiable risk factor for cardiovascular disease, the most common cause of morbidity and mortality worldwide (Whelton, 1994; Wilson, 1994; Yusuf et al., 2001; Ezzati et al., 2002; WHO, 2013). Chronically elevated BP potentiates the risk of stroke, heart and renal failure and cardiac events (Whelton, 1994; Kearney et al., 2005). Since 2010 it has been estimated that hypertension contributes to more than 9.4 million deaths and 7% of global DALYs (Disability-Adjusted Life Burden as a representation of the numbers of years lost due to ill health or early death) annually, and was the single greatest risk factor globally for these (Lim et al., 2012). It is estimated that 30–40% of the world's population suffers from hypertension, although the true incidence may be higher as elevated BP can remain asymptomatic (Lawes et al., 2008; Roger et al., 2012; Mancia et al., 2013). The prevalence of hypertension increases with ageing; whilst it has been estimated that fewer than 10% of people under the age of 40 may have elevated BP, more than 60% of people aged over 70 require clinical intervention for hypertension (Lloyd-Jones et al., 2005; Egan et al., 2010). The world's population is ageing; the number of people over 60 years of age has doubled since 1980 and is predicted to reach 2 billion by 2050 (WHO, 2014). A consequence of this is an increased number of people living with hypertension and associated complications. Indeed, it has been estimated that between 1980 and 2008 the global number of people with hypertension rose from 600 million to almost 1 billion (Danaei et al., 2011), and that this number is continuing to rise (Fields et al., 2004; Heidenreich et al., 2011).

The relative risk for cardiovascular disease is directly associated with the absolute level of BP (Whitworth, 2003), with the risk of cardiovascular disease having been reported to double for each 20/10 mmHg increment increase in BP (Chobanian et al., 2003). Treatments for hypertension often involve a combination of lifestyle changes and pharmacological intervention and, when successful, can have a highly positive impact on cardiovascular risk. Data from randomised controlled trials and prospective studies have shown that lowering BP by even 5–6 mm Hg is associated with approximately 20% and 40% reduction in adverse coronary events and stroke respectively (Lewington et al., 2002; Law et al., 2009). However, some current therapeutic strategies appear to be of limited effectiveness for an increasing number of patients, with multidrug resistance or poor adherence to treatment regimes being reported (Morisky et al., 2008; Tomaszewski et al., 2014; Vega & Bisognano, 2014; Vongpatanasin, 2014; Sakhuja et al., 2015). Resistant hypertension is associated with increased target organ damage and cardiovascular complications (Muiesan et al., 2013). As such, and alongside likely increasing demand from an increasingly ageing global population, there is a pressing need for development of new and more effective strategies to prevent and treat hypertension. Development of such strategies relies on a continued evolution of our understanding of the mechanisms which control BP.

2.2. Factors contributing to the development of hypertension

Although high BP may be attributable to clear medical conditions, termed secondary hypertension, it remains that no clear cause is evident for around 90% of people who have high BP (Lloyd-Jones et al., 2005; Messerli et al., 2007). This type of hypertension is called primary or essential hypertension. It has long been accepted that BP is a quantitative trait with continuous variation, pointing to multiple factors influencing the level of human BP (Pickering, 1972; Deng, 2007). It is clear that lifestyle can have a very important impact on hypertension and cardiovascular health, and, as there are numerous publications in this area, this will not be considered further in this review. However, it is also apparent that BP has a heritable component of about 15–60%; such a range being dependent on whether in-clinic or ambulatory measures of BP are studied (Mongeau, 1989; Kotchen et al., 2000; Kupper et al., 2005; Zuk et al., 2012). Whilst, from a pharmacological point of view, it is important to understand the underlying molecular mechanisms which contribute to the development of essential hypertension, this is confounded by the complex nature in which potentially multiple susceptibility genes are themselves modulated by environmental factors and interactions with other genetic loci (O'Shaughnessy, 2001).

Recent advances in genetic sequencing technology have enabled investigations to determine whether specific genetic factors can confer susceptibility to disease. Genome wide association studies (GWAS) in multiple different ethnic populations have revealed at least 52 distinct genetic variants with significant association to BP and/or hypertension (McCarthy et al., 2014; Tragante et al., 2014). Each individual variant appears to only have a modest direct effect on BP (less than 1mmHg per allele for systolic pressure), so, even collectively, these loci may only provide a minor contribution to the known percentage heritability of BP (Johnson et al., 2011; McCarthy et al., 2014). Therefore, it appears more genes, pathways or gene–environment interactions remain to be elucidated to more fully describe the genetic basis of essential hypertension. Whilst such associations can highlight new targets for possible regulation of BP, physiologically testing the function of genes within target loci is a key requirement for confirming whether a gene within, or part of, a loci of association is indeed part of a pathway which can influence BP.

3. Resistance arteries and essential hypertension

BP is the product of cardiac output and total peripheral resistance. Although cardiac output and sympathetic activity may be elevated in the early stages of hypertension, increased peripheral resistance is the main driver for chronically elevated BP (Heagerty et al., 2010). Resistance to flow can be influenced by blood viscosity, vessel length and vessel diameter, with resistance being inversely proportional to the fourth power of the vessel's radius (Poiseuille's law) (Badeer, 2001; Sriram et al., 2014). Small arteries which provide the greatest percentage contribution to peripheral resistance have been termed resistance arteries, and are defined as pre-capillary vessels composed of a relatively thick media layer and having internal diameter of approximately 100–300 μm (Mulvany & Aalkjaer, 1990; Korsgaard et al., 1993; Christensen & Mulvany, 2001; Heagerty et al., 2010). As resistance

arteries play a key role in regulating peripheral vascular resistance, the structure and contractility of these vessels are crucially important factors in determining and regulating BP. Importantly, changes in both the structure and function of resistance arteries are believed to play an essential role in the development and progression of hypertension (Heagerty et al., 2010; Rizzoni & Agabiti-Rosei, 2012).

3.1. Resistance artery structure and hypertension

It is well established that structural changes in resistance arteries are associated with chronically elevated BP (Heagerty et al., 2010; Rizzoni et al., 2011; Mulvany, 2012). There is now significant evidence to support the notion that high BP is associated with a remodelling of resistance arteries such that there is a thickening of the medial layer of the arterial wall of resistance arteries, a reduced lumen diameter and thus an increased media to lumen ratio (Korsgaard et al., 1993; Martinez-Lemus et al., 2009; Rizzoni & Agabiti-Rosei, 2012; Renna et al., 2013). Although growth may be evident in certain pathologies and in advanced hypertension (Rizzoni et al., 1996), there is now a wealth of evidence to suggest that inward remodelling of resistance arteries with no significant change in cross sectional area, termed eutrophic remodelling, is a key feature of essential hypertension, and underpins elevated peripheral resistance (Heagerty et al., 1993; Izzard et al., 2005; Heerkens et al., 2006; Staiculescu et al., 2013). Eutrophic remodelling of resistance arteries is proposed to be a consequence of realignment and potentially closer associations between vascular smooth muscle cells (VSMCs) of the arterial wall, and not hypertrophy and/or hyperplasia of VSMCs, generating a detectable narrowing in lumen diameter of the vessel (Heagerty et al., 2010), Fig. 1.

There are now numerous studies which support the notion that changes in small artery structure have strong prognostic significance in hypertensive patients, over and above all other known cardiovascular risk factors (Rizzoni et al., 2003; De Ciuceis et al., 2007; Mathiassen, Buus, et al., 2007b). Understanding the mechanisms which may modulate resistance arterial eutrophic remodelling is thus important, but these remain poorly understood. Prolonged vasoconstriction appears important, which, in itself may modulate re-organisation via the activities of cross-linking enzymes such as tissue-type transglutaminase and/or integrins (Bakker et al., 2002, 2005; Heerkens et al., 2006). It has been proposed that initial eutrophic remodelling may be an energetically favourable adaptive mechanism to normalise wall stress to maintain adequate blood flow, whilst a growth response, which may be observed in advanced or secondary hypertension, is related to a greater adverse prognosis (Izzard et al., 2006; Heagerty et al., 2010).

It is important to note that in essential hypertension the relationship between BP and remodelling remains unclear. Whilst some studies suggest that structural changes follow a change in BP and/or prolonged arterial vasoconstriction (Bakker et al., 2002), there is evidence from studies using genetically modified mice that structural changes may precede the development of hypertension (Zacchigna et al., 2006). Whilst studies on isolated arteries from animal models of hypertension suggest that remodelling may be a consequence of reduced resistance arterial distensibility (at least in the cerebral circulation) (Izzard et al., 2006), others have shown increased distensibility (Laurant et al., 1997). Thus, there is little to support changes in distensibilty of resistance arteries being the primary driver for remodelling in all vascular beds. Therefore, debate on this topic continues; hence, further understanding of how arterial remodelling may be initiated and progresses in relation to a change in BP is important for elucidating the factors contributing to the development and progression of high BP.

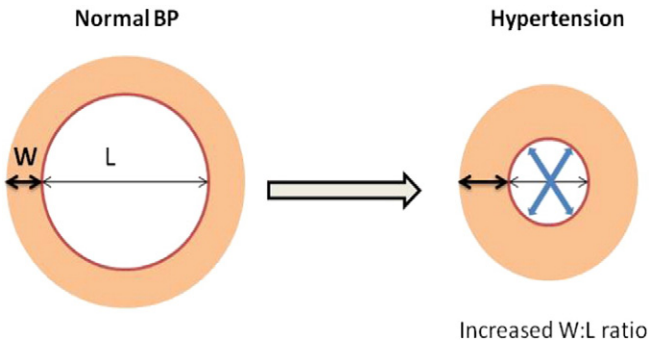

Fig. 1. Eutrophic remodelling of resistance arteries. Increased intraluminal pressure (⬇) is associated with cellular rearrangement, principally of VSMCs, resulting in a narrower lumen diameter (L) and increased wall thickness (W) of the vessel.

3.2. Arterial contractility and hypertension

The contractility of resistance arteries is important in setting peripheral vascular resistance, and thus BP. Whilst enhanced arterial constriction will, in itself, increase vascular resistance, it appears that the major consequence of altered levels of contractile activation in hypertension is on arterial remodelling. Studies on isolated rat small arteries maintained in culture have shown that chronic vasoconstriction itself induces eutrophic remodelling and that this remodelling is independent of the vasoconstrictor stimulus (Bakker et al., 2000, 2002, 2004). Furthermore, chronic in vivo infusion of vasoconstrictor stimuli in animals has also been shown to induce remodelling (Eftekhari et al., 2007). Enhanced contraction may occur as a result of changes in external stimuli and/or due to underlying contractile mechanisms. Although elevated sympathetic activity, increased sensitivity to vasoactive stimuli (Angus et al., 1992) and increased myogenic tone (Izzard et al., 1996) have been shown to be involved in the development of hypertension in some vascular beds, such changes do not appear to persist when hypertension is established (Izzard et al., 1996, 2006). There is much debate in the literature about whether structural changes amplify vasoconstrictor responses (Wright & Angus, 1999; Folkow, 2000; Korner et al., 2000; Izzard et al., 2002), hence the relationship between active tone and remodelling remains to be fully elucidated. Nevertheless, it is generally accepted that changes in contractility of some resistance arteries play a key role in remodelling during the development of hypertension. In support of this it has been shown that normalisation of vascular structure by anti-hypertensive therapies was independent of BP but was dependent on vasodilatation (Mathiassen, Buus, et al., 2007a; Mulvany, 2012). Thus, it is important to further understand the mechanisms which modulate resistance arterial contractility in both the developing and sustained phases of hypertension.

Arterial tone is principally regulated via the contractile state of VSMCs. This can be modulated by stimuli acting directly on the VSMCs or by those acting on the vascular endothelium which lines the lumen of blood vessels. The endothelium modulates arterial tone by the release of and/or stimulation of powerful vasodilating and vasoconstricting factors, principally nitric oxide (NO), prostacyclin and endothelium derived hyperpolarising factor (Furchgott & Zawadzki, 1980; Mombouli & Vanhoutte, 1999; Feletou & Vanhoutte, 2009). NO is a powerful vasodilator and is synthesised via nitric oxide synthase (NOS) of which three forms exist; endothelial NOS (eNOS) primarily from endothelial cells; neuronal NOS (nNOS) which is expressed in peripheral nitrergic nerves innervating smooth muscle and also reported in aortic VSMCs; and inducible NOS (iNOS)—the source of which is not clear in the vasculature ((Forstermann et al., 1995; Furchgott & Zawadzki, 1980); H. (Schwarz et al., 1999; Li et al., 2002)). NO induces dilation via activation of cyclic guanosine monophosphate (cGMP)-mediated pathways (Waldman & Murad, 1987; Bolotina et al., 1994; Ignarro, 2002). cGMP appears to be important in BP regulation as global ablation of cGMP results in increased conscious BP (Pfeifer et al., 1998).

Endothelial dysfunction has been reported in numerous disease states including in hypertensive rodents and has also been proposed to occur in old hypertensive patients (Fujii et al., 1992; Taddei et al.,

1997; Vanhoutte et al., 2005; Taguchi et al., 2015), however, this is not a uniform finding (Li & Bukoski, 1993). Indeed, in some studies endothelium-dependent relaxation of resistance arteries has been reported to be preserved in hypertension by an NOS-dependent mechanism (Kang et al., 2007). Other studies have shown that abnormal endothelial function in small blood vessels does not predict cardiovascular events in patients with essential hypertension (Rizzoni et al., 2006) and indeed such dysfunction appears to be independent of vascular structural changes in hypertension (Rizzoni et al., 1998). There continues to be debate as to the role of the endothelium in the development and progression of hypertension (Luscher, 1994), nevertheless, the endothelium is an important modulator of vascular contractility and BP which should not be ignored when developing new therapeutic targets for hypertension.

3.3. Mechanisms controlling arterial contractility; role of intracellular free calcium

It is ultimately the contractile state of vascular smooth muscle which determines the level of vascular tone. As such, it is important to understand the underpinning mechanisms which mediate smooth muscle contractility.

The concentration of intracellular free calcium ($[Ca^{2+}]_i$) underpins the modulation of VSMC contractility and thus arterial diameter and vascular resistance. An increase in $[Ca^{2+}]_i$ results in activation of myosin light chain kinase (MLKC), subsequent phosphorylation of myosin, and VSM contraction. Conversely, a fall in $[Ca^{2+}]_i$ results in activation of myosin light chain phosphatase and dephosphorylation of myosin, hence promoting arterial relaxation (Somlyo & Somlyo, 1994; Gollasch et al., 2000; McCarron et al., 2006). $[Ca^{2+}]_i$ levels are modulated by either influx/efflux across the plasmalemma and or release/sequestration into intracellular calcium stores predominantly, but not exclusively, the sarcoplasmic reticulum (SR). (Although a brief overview of the main mechanisms is given below a detailed discussion of the mechanisms which modulate calcium homeostasis in smooth muscle is beyond the scope of this text and as such we direct the reader towards reviews of this area for more information (Amberg & Navedo, 2013; Hill-Eubanks et al., 2011; Kudryavtseva et al., 2013)). The relationship between Ca^{2+} and contraction is, however, not straightforward and it is now evident that subcellular Ca^{2+} fluxes and microdomains exist which may differentially modulate contractile function (Gollasch et al., 2000; McCarron et al., 2006). Whilst an increase in global $[Ca^{2+}]_i$ promotes arterial contraction, localised increases in Ca^{2+} may have different effects on contraction. For example, in isolated myogenic cerebral arteries, local increases in intracellular calcium following activation of ryanodine receptors on the SR cause arterial vasodilation via activation of Ca^{2+}-activated potassium channels (Nelson et al., 1995). In contrast, in isolated retinal arteries it has been shown that Ca^{2+} sparks can promote myogenic tone, most likely via summation to generate Ca^{2+} waves/oscillations (Tumelty et al., 2007; Kur et al., 2013). Although modulation of the calcium sensitivity of the contractile apparatus can regulate VSMC contractility (Kimura et al., 1996; Uehata et al., 1997; Andre et al., 2014; Takeya et al., 2014) it is clear that regulating $[Ca^{2+}]_i$ within VSMCs plays a crucial role in regulating the contractile state of VSMCs, and hence vascular resistance and thus BP. As such, factors which modulate Ca^{2+} homeostasis in vascular smooth muscle are particularly attractive therapeutic targets for BP reduction and potential reversal of inward remodelling in resistance arteries.

Ca^{2+} influx into VSMCs is principally driven by plasma membrane voltage gated Ca^{2+} channels (VGCC), particularly L type Ca^{2+} channels. Though, notably, increased $[Ca^{2+}]_i$ can be promoted by activation of Ca^{2+} release channels, including ryanodine receptors and inositol 1,4,5-trisphosphate receptors on the SR, and also via transient receptor potential (TRP) channels (Jackson, 2000; Parekh & Putney, 2005; Earley et al., 2007; Gonzalez-Cobos & Trebak, 2010). There is evidence to suggest that high BP can modulate activation of calcium channels via changes in their expression level or by affecting their functional ionic conductance (Stekiel et al., 1986; Pesic et al., 2004). Such effects can exacerbate vasoconstriction and elevate vascular resistance and BP; hence, treatment strategies to reduce high BP have long involved the use of L-type VGCC inhibitors (Sonkusare et al., 2006). Further, Ca^{2+} channel blockers which dilate resistance arteries have been shown to reverse eutrophic remodelling (Schiffrin, 2004; Agabiti-Rosei & Rizzoni, 2010). Taken together this suggests that therapies which target intracellular calcium homeostasis are worthy of exploration. This should also include investigation into mechanisms which reduce levels of intracellular Ca^{2+} and could stimulate arterial dilation. These mechanisms include sequestration into intracellular stores, most notably into the SR via its Ca^{2+}-ATPase pump (SERCA), and via extrusion across the plasma membrane. The activity of SERCA is regulated by phospholamban (PLN); aortic contractions from PLN-deficient mice have been shown to be reduced, effects which were abolished by pharmacological SERCA inhibitors (Lalli et al., 1997; Oloizia & Paul, 2008). Thus, modulation of mechanisms which decrease cytoplasmic Ca^{2+} can modulate arterial contractility and may thus be a potential target for reduction of BP. Ca^{2+} can be extruded out of VSMCs by two different Ca^{2+} transporting systems, the Na^+/Ca^{2+} exchanger (NCX), predominantly type 1 (NCX1) (Quednau et al., 1997), and plasma membrane calcium ATPase type 1 or 4 (PMCA1, PMCA4) (Oloizia & Paul, 2008). In the heart, NCX plays a major role in Ca^{2+} extrusion extruding one Ca^{2+} out of the cell in exchange for three Na^+ ions (Bers, 2000). In VSMCs, however, it is bi-directional and can modulate Ca^{2+} efflux or influx depending on the relative concentrations of Ca^{2+} and Na^+ (Blaustein & Lederer, 1999) and its role in Ca^{2+} extrusion has been questioned (Kamishima & McCarron, 1998). Indeed smooth muscle-specific genetic ablation of NCX1 lowers BP (Zhang et al., 2010; Zhao et al., 2011), whilst, conversely, mice overexpressing NCX1 in smooth muscle display increased BP (Blaustein & Lederer, 1999; Iwamoto et al., 2004); effects not consistent with a predominant role in Ca^{2+} extrusion. Currently there is little known about the specific contribution of PMCAs to Ca^{2+} efflux in VSM, but in bladder and myometrial smooth muscle extrusion by this route has been reported to contribute approximately 30% and 70% of total calcium efflux respectively (Matthew et al., 2004; Liu et al., 2006). Therefore, a greater understanding of the role of PMCAs in the microcirculation is important for further understanding of the regulation of BP and how it might be pharmacologically modulated.

4. Plasma membrane calcium ATPases

PMCAs are 10 transmembrane domain ATP-dependent pumps of the P-type family. Four separate PMCA proteins, 1–4, are evident in mammals with each being expressed from its respective gene (i.e. *ATP2B1* gene encodes PMCA1 protein). Numerous splice variants of each gene have been found to be functionally expressed, with the possibility that over 30 variants can be generated (Keeton et al., 1993; Di Leva et al., 2008). The N and C termini of PMCA are both intracellular, with the far longer C terminus containing regulatory domain binding sites and PDZ domains physically linking to interacting partners (James et al., 1988; Kessler et al., 1992; Hofmann et al., 1993; Di Leva et al., 2008). Extracellular loops are believed to be of minimal length (Di Leva et al., 2008), Fig. 2.

PMCA1 is ubiquitously expressed in humans; ablation of PMCA1 is embryonically lethal early in mouse development leading to the suggestion that the protein has a 'housekeeping' or developmental role (Okunade et al., 2004; Brini, 2009; Kenyon et al., 2010). PMCA4 is also present in most cell types, but its function may be more tissue specific than PMCA1 (Schuh et al., 2004). Tissue expression of PMCA2 and PMCA3 is far more limited, with their main functional role being in neuronal cells and the ear (Kozel et al., 1998). PMCA1 and PMCA4 are both present in the vasculature and thus have the potential to contribute to vascular structure and function.

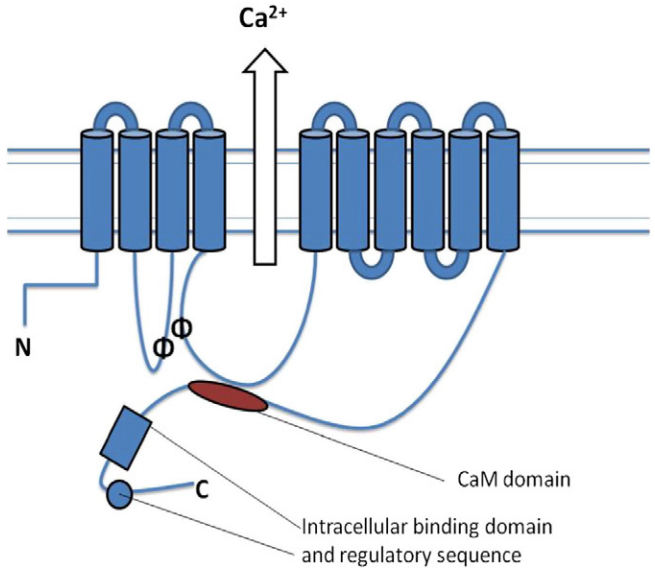

Fig. 2. Schematic representation of the transmembrane positioning of the PMCA protein. The 10 transmembrane domain structure of mammalian PMCA. N and C termini for both PMCA1 and 4 are intracellular. ATP binding to the catalytic domain (Φ) promotes Ca^{2+} efflux, which is modulated by the autoinhibitory Ca^{2+}-calmodulin binding domain (CaM).

4.1. Expression of plasma membrane calcium ATPases in the vasculature

PMCAs have been shown to be present in both VSMCs and endothelial cells and can thus potentiate vascular function via actions on either of these cells types. PMCA1 RNA has been detected in pig aortic smooth muscle and endothelial cells (Pande et al., 2006), whilst mRNA for PMCA1 has also been detected in rat cultured aortic VSMCs (Sasamura et al., 2002). At the protein level, PMCA1 is present in VSMCs from mouse aorta (Kobayashi et al., 2012), as well as in endothelial cells from the pig coronary artery (Szewczyk et al., 2007).

PMCA4 mRNA has been detected in endothelial and VSMCs of porcine aorta (Szewczyk et al., 2007), in VSMCs from mouse carotid artery (Afroze et al., 2014), and was shown as the most prominent PMCA mRNA transcript in mouse whole aorta (Okunade et al., 2004). PMCA4 protein expression has been reported in a mouse VSMC line (Afroze et al., 2003) and in isolated murine aortic VSMCs (Momen et al., 2014).

Although it is clear that both PMCA1 and PMCA4 are present in the vasculature their relative expression is difficult to quantify. However, Pande and colleagues have suggested that PMCA4 is the dominant form in pig aortic VSMCs (Pande et al., 2006). In contrast, others have shown that mRNA for PMCA1 is more prevalent than that for PMCA4 in rat cultured aortic VSMCs, with PMCA4 transcripts suggested to be at only a quarter of the level of those for PMCA1 (Sasamura et al., 2002). In porcine aortic endothelial cells PMCA1 RNA has been shown to be detectable at a higher level than PMCA4 transcripts (Pande et al., 2006; Szewczyk et al., 2007). Thus, the vascular expression of PMCA1 and 4 may be species specific, with further work being required to clearly determine the relative expression of PMCA1 and PMCA4 in VSMCs and endothelial cells, particularly in resistance arteries.

5. Plasma membrane calcium ATPase 1, arterial contractility and blood pressure regulation

Although multiple factors underpin the development of essential hypertension, heritability of 30–60% may combine with other risk factors to potentiate disease development (Miall & Oldham, 1963; Kupper et al., 2005). As such, there has been a long standing search for genetic components of hypertension. Advances in genome sequencing technology, determining quantitative trait loci by assessing single nucleotide polymorphisms (SNPs) in disease relative to control cohorts, have, relatively recently, highlighted genomic regions which may contribute as single factors in BP regulation. Crucially, a genomic region containing *ATP2B1*, which encodes for PMCA1, has been identified as having a very strong association with BP variance and/or with hypertensive disease in different human population groups (Cho et al., 2009; Levy et al., 2009; Hong et al., 2010; Takeuchi et al., 2010; Johnson et al., 2011). Importantly, SNPs associating *ATP2B1* with BP have attained genome wide significance in replication analyses in multiple different ethnic groups and have shown a consistently high level of genetic association (Tabara et al., 2010; Miyaki et al., 2012; Xi et al., 2012; Ganesh et al., 2013; Wang et al., 2013). SNPs relating to *ATP2B1* in a Japanese cohort were found to give an odds ratio of 1.17 to 1.31 for hypertension (Tabara et al., 2010), a very similar level of risk as reported by other large scale GWAS. More recently *ATP2B1* has been genetically linked with pulse pressure (Kelly et al., 2013) and to coronary artery calcification and myocardial infarction in chronic kidney disease (Ferguson et al., 2013), further substantiating a possible genetic link to cardiovascular function. How *ATP2B1* may be associated with cardiovascular function cannot be directly determined by GWAS. Detection of SNPs associated with hypertension in the promoter region of *ATP2b1* (Cho et al., 2009; Tabara et al., 2010) could suggest that it is modulation of gene expression which may drive a BP phenotype. Furthermore, *ATP2b1* mRNA expression has been reported to be reduced in human umbilical artery smooth muscle cells from people carrying risk alleles as determined by GWAS (Tabara et al., 2010). However, with *ATP2b1* genomic regions also having been shown to be associated with hyperlipidemia and diabetes (Heo et al., 2014), the potential role of PMCA1 in BP regulation may additionally be due to indirect effects on metabolism.

Thus, there is accumulating evidence to support an important role for *ATP2b1* in BP regulation. It should, however, be noted that genome linkage analysis is correlative and does not definitively demonstrate a cause-effect relationship between a genomic region and a BP phenotype (Deng, 2007). Therefore, it has been paramount to physiologically link PMCA1 to a BP phenotype to clearly show the protein can indeed functionally regulate BP.

Genetically modulated animal models are widely used to directly investigate the effect of changes in gene and protein expression on physiological function. Global ablation of *Atp2b1* in mice is embryonically lethal early in development, between 3.5 and 8.5 days of pregnancy, suggesting an important role for PMCA1 in development (Okunade et al., 2004; Shaheen, 2012). PMCA1 has been proposed to have a role in BP regulation as mice bred to be globally heterozygous for *Atp2b1*, whilst indeed viable, exhibit elevated BP (Fujiwara et al., 2014). Elevated peripheral BP was also reported in mice following injection of siRNA constructs to block production of PMCA1 protein (Shin et al., 2013). Studies using mice with VSMC specific deletion of PMCA1, generated using the Cre-loxP system, support a specific role in the vasculature for *Atp2b1*. Whilst the gross phenotype and survival of these mice were unaffected, they did exhibit significantly elevated systolic BP (Kobayashi et al., 2012).

Femoral arteries from mice devoid of VSM PMCA1 protein exhibited enhanced contractility to phenylephrine (Kobayashi et al., 2012), whilst studies using heterozygous *Atpb1* mice demonstrated both an increased contractility to phenylephrine and a reduced endothelium-dependent dilation to acetylcholine from isolated abdominal aorta vascular rings (Fujiwara et al., 2014). Similarly, elevated BP following silencing of *Atp2b1*, has been associated with enhanced isolated small artery contractility to phenylephrine and increased myogenic responsiveness (Shin et al., 2013). Importantly, siRNA knockdown of *Atp2b1* has been associated with an increased media:lumen ratio in mesenteric resistance arteries, characteristic of essential hypertension (Shin et al., 2013). Thus there is now substantial evidence to support an involvement of PMCA1 in BP regulation, in part at least, via effects on the structure and function of small arteries.

The mechanisms whereby modifications in the expression of *Atp2b1*/PMCA1 can modulate arterial structure and function still require further elucidation; however, as PMCA1 is a Ca^{2+} extrusion pump one potential way is clearly by modulation of global $[Ca^{2+}]_i$. In support of this, cultured VSMCs from smooth muscle-specific PMCA1 deleted mice exhibited elevated levels of basal $[Ca^{2+}]_i$ and also in response to phenylephrine stimulation (Kobayashi et al., 2012). However, evidence from heterozygous PMCA1 mice shows that PMCA1 can also modulate arterial tone via effects on NO, effects attributed to a reduced phosphorylation of eNOS (residue Ser-1177) and production of NO (Fujiwara et al., 2014). Endothelial cells from heterozygous Atp2b1 mice exhibit reduced expression of eNOS, with no significant difference in nNOS expression being reported (Fujiwara et al., 2014). A functional interaction between PMCA1 and eNOS has been demonstrated in human endothelial cells, however, in contrast to animal studies, PMCA1 appears to be a negative regulator of eNOS primarily via increased phosphorylation of Thr-495 of eNOS; Thr-495 being an inhibitory residue (Holton et al., 2010). There is clearly a need for further study to elucidate the effects of PMCA1 on eNOS activity, endothelial function and subsequent effects on the resistance vasculature and on BP. Also It should be noted that the effects of PMCA1 on other endothelial factors also needs to be established especially as NO generally plays a smaller role in resistance arteries than it does in large arteries (Urakami-Harasawa et al., 1997).

Thus, there is now increasing evidence for a clear link between *Atp2b1*/PMCA1 and BP, effects which can be modulated by changes in arterial contractility. Effects of PMCA1 in VSMCs and on global $[Ca^{2+}]_i$ are consistent with a major role for PMCA1 in Ca^{2+} extrusion, whilst how PMCA1 may be involved in arterial remodelling requires further investigation. Further work is clearly required to elucidate the different ways in which PMCA1 may modulate arterial contraction. To date, data on only one relatively selective inhibitor of PMCA1, the small peptide caloxin 1b3, has been published (Szewczyk et al., 2010). Caloxin 1b3 has been reported to be selective for PMCA1 in rabbit duodenal mucosa and pig coronary artery endothelial cells, and has been shown to increase $[Ca^{2+}]_i$ in these endothelial cells (Szewczyk et al., 2010). However, the influence of caloxin 1b3 on arterial contractility and BP remains to be clearly determined. Nevertheless, the strong clinical evidence of an association between *ATP2B1* and BP, coupled with experimental studies directly linking changes in PMCA1 expression to increases in resistance arterial contractility and increased BP, make PMCA1 a very attractive potential therapeutic target which warrants further exploration.

6. Plasma membrane calcium ATPase 4 in blood pressure regulation

Although there is a wealth of clinical evidence suggesting a role for PMCA1 in BP regulation there is also substantial evidence from experimental studies which show that changes in the expression of PMCA4 can also modulate arterial contractility and BP. To date, two investigative approaches have been taken; 1) studies employing transgenic overexpression of PMCA4 in vivo and 2) pharmacological inhibition of PMCA4.

6.1. Transgenic expression of plasma membrane calcium ATPase 4

Studies conducted by Gros and colleagues and Schuh and colleagues over a decade ago provided the first evidence that PMCAs can modulate arterial contractility and BP (Gros et al., 2003; Schuh et al., 2003). Both groups generated transgenic mice overexpressing human PMCA4b, either under control of vascular-specific SM22α promoter or doxycycline-induced overexpression in smooth muscle, (Gros et al., 2003; Schuh et al., 2003). Perhaps surprisingly, both transgenic mouse models exhibited an increase in BP. The elevated BP was accompanied by enhanced aortic contraction to K^+-mediated membrane depolarisation (Schuh et al., 2003), plus heightened myogenic tone and elevated response to adrenergic stimulation in mesenteric arteries (Gros et al., 2003). These effects are not consistent with PMCA4 acting as a major contributor to removal and modulation of global $[Ca^{2+}]_i$. Indeed, it has been shown that basal arterial VSMC $[Ca^{2+}]_i$ was comparable in both PMCA4 overexpressing and control mice (Gros et al., 2003). The changes in arterial contractility in both animal models were prevented by inhibition of nNOS pointing to negative regulation of nNOS activity by PMCA4 (Gros et al., 2003; Schuh et al., 2003). Previous studies in human endothelial cells have shown that PMCA4 interacts with eNOS leading to increased phosphorylation at Thr-495 and reduced enzymatic activity (Holton et al., 2010). However, in aortae at least, endothelium-dependent relaxation to acetylcholine was unaffected by overexpression of PMCA4 (Schuh et al., 2003). Although it is important that the effects of PMCA4 overexpression on endothelial function in resistance arteries are investigated, results so far, together with the fact that NO plays a lesser role in endothelium-dependent dilation in resistance arteries when compared to large arteries (Urakami-Harasawa et al., 1997), suggest that the endothelium does not play a major role in the effects of PMCA4 on modulation of arterial contraction and BP. To date, no published studies have looked at the effects of genetic ablation of *Atp2b4* (for PMCA4 protein) on resistance arterial structure or function.

It is now evident that PMCA4 has a major role in signal transduction in a number of excitable cells. In isolated molecular experiments, cardiomyocytes and whole heart tissue PMCA4 functionally associate with calcineurin (Buch et al., 2005), Ras-associated factor 1 (Armesilla et al., 2004), α1-syntrophin (Williams et al., 2006) and nNOS, the latter via interaction with the PDZ domain on PMCA4 (Schuh et al., 2001; Oceandy et al., 2007; Duan et al., 2013). In blood vessels any such association of PMCA4 and nNOS clearly appears to be of relevance to the regulation of arterial contractility. It is well known that NO can influence contractility of peripheral blood vessels, and it is becoming more apparent that vascular NO production is not just mediated by eNOS but also via nNOS activity (Kaley et al., 1992; Buchwalow et al., 2002; Webb et al., 2006). nNOS-mediated NO production in a cellular assay has been shown to be significantly reduced when the enzyme is tethered as part of a macromolecular complex including PMCA4, confirming that PMCA4 is a negative regulator of nNOS activity (Schuh et al., 2004). The exact way in which PMCA4 modulates vascular nNOS activity is unknown, but nNOS activity is calcium/calmodulin dependent and a role for PMCA4 in regulating localised Ca^{2+} concentrations in a microdomain has been suggested (Cartwright et al., 2007). More recent investigation of the role of PMCA4 in cardiac signalling has supported this supposition, pointing to PMCA4 having an important role as a structural molecule in the heart (Oceandy et al., 2007; Mohamed et al., 2011). Overexpression of PMCA4 in arterial smooth muscle has been shown to significantly reduce nNOS activity (Gros et al., 2003). Hence, reduced production of vasodilatory NO has been proposed in explanation for elevated BP observed in PMCA4 overexpressing mice (Gros et al., 2003; Schuh et al., 2003). Thus, studies from transgenic overexpressing mice suggest that PMCA4 is a powerful modulator of BP and that changes in resistance arterial contractility, as a result of negative regulation of nNOS by PMCA, are likely contributors to this. However, a different role for PMCA4 in the vasculature has been proposed following a different experimental approach: that of pharmacological inhibition of PMCA4 using caloxin peptides (Chaudhary et al., 2001).

6.2. Pharmacological modulation of plasma membrane calcium ATPase 4 activity

The role of PMCA4 in the vasculature has been further assessed pharmacologically by using caloxin peptides, which have been designed to inhibit the protein's ability to pump Ca^{2+}. A number of caloxin compounds have been designed, with screens against PMCA4 identifying caloxin 1b1 as an inhibitor of PMCA4 ($K_i = 46 \pm 5$ μM compared $K_i = 105 \pm 11$ μM for PMCA1) (Pande et al., 2006). By binding to the

first extracellular domain of PMCA4 it is proposed that caloxin 1b1 inhibits Ca^{2+} flux non-competitively by blocking/altering the required conformational change in the protein which occurs with the binding of ATP (Holmes et al., 2003; Pande et al., 2006).

Although the effects of caloxin 1b1 on BP have not been investigated, acute application has been shown to increase contraction of rat aortic rings in response to agonist stimulation (Pande et al., 2006). Furthermore, caloxin 1b1 has been shown to potentiate increased $[Ca^{2+}]_i$, promoted by Ca^{2+} ionophore application in cultured arterial VSMCs derived from porcine aorta (Pande et al., 2006). Subsequently caloxin 1c2 ($K_i = 2.3 \pm 0.3$ μM), a more selective PMCA4 inhibitor with ten-fold greater selectivity for PMCA4 over other PMCAs, has been shown to increase the basal tone of isolated coronary arteries and the force of their contraction compared to control tissues at low extracellular Ca^{2+} levels ($[Ca^{2+}]o$) (<1.6 mM) whilst in the presence of inhibition of NCX and SERCA (Pande et al., 2008). Thus, available evidence to date as to the effects of caloxins correlates with the expectation that inhibiting a Ca^{2+} extrusion pump would elevate $[Ca^{2+}]_i$ in PMCA4 expressing VSMCs and contribute to augmented vascular contractility and BP. It is however important to highlight that previous studies have only explored the effects of caloxins on larger arteries, namely coronary arteries and the aorta, which do not make significant contributions to overall vascular resistance and BP (Pande et al., 2006, 2008). There is thus a pressing need to explore the effects of PMCA4 inhibitors on resistance arteries. The effects of PMCA4 inhibition on endothelium function also need to be elucidated.

As previously discussed, regulation of $[Ca^{2+}]_i$ in vascular cells is important for determining arterial tone and vessel contractility. Whilst the effects of caloxins point to a role for PMCA4 in BP regulation, inhibiting the Ca^{2+} extrusion pumping capability of PMCA4 seems inappropriate for a novel direct anti-hypertensive drug. However, other factors relating to tissue perfusion may also be considered, as PMCA4 has been proposed as an endogenous regulator of processes leading to angiogenesis (Baggott et al., 2014). Hence, there is a requirement for further understanding of the effects of inhibiting PMCA4 and also development of new drugs against the protein.

We have recently identified aurintricarboxylic acid (ATA) as a novel inhibitor of PMCA4, being capable of fully inhibiting PMCA4 at 1 μM (Mohamed et al., 2013). In vitro, ATA was found to have a dose dependent effect on PMCA4 (IC_{50} approximately 150nM), and have only a very minor effect on PMCA1 and SERCA with no inhibitory effect on the Na^+/K^+ ATPase (Mohamed et al., 2013). Thus, we propose that ATA, with its high level of selectivity over other PMCA isoforms and P-type ATPases, will be a useful tool to further elucidate the effects of PMCA4 in regulating resistance arterial contractility and BP. ATA, however, modulates a variety of different cellular processes. For example, it has been shown to act as a nuclease inhibitor for isolation of nucleic acids (Hallick et al., 1977; Gonzalez et al., 1980), as an inhibitor of the RISC complex hence blocking micro RNA synthesis (Tan et al., 2012) and as a potential inhibitor of glutamate dehydrogenase (Li et al., 2007). It has also been shown to protect cells from human complement-mediated lysis, to reduce tube formation by endothelial cells (Lipo et al., 2013) and to have neuroprotective effects, possibly via inhibition of complement factors of the innate immune system (Lee et al., 2012) or via promoting receptor tyrosine kinase signalling (Okada & Koizumi, 1995). Whether these effects are due, in part at least, to the effects of ATA on PMCA4 has largely not been considered. Whilst multiple actions of ATA may limit its use as a therapeutic cardiovascular strategy, we have previously discussed that complete inhibition of PMCA4 can be achieved at 1 μM, a concentration approximately 100 fold lower than which effects on other targets have been reported to occur (Mohamed et al., 2013). Nevertheless, the development of more specific pharmacological tools for PMCA4 is essential to further our understanding of the effects of PMCA4 on resistance arterial contraction and BP.

In summary, available evidence to date suggests that both overexpression and inhibition of PMCA4 increase arterial contractility which may, in turn, increase BP. Whilst it is clear that PMCA4 affects arterial contractility and BP, these effects, and indeed the underpinning mechanisms, may be complex and variable and many questions remain unanswered. Studies to date suggest that PMCA4 may modulate bulk Ca^{2+} homeostasis, nNOS signalling (Pande et al., 2006, 2008) and eNOS activity (Holton et al., 2010). It is noteworthy that recent studies investigating PMCA4 in the cell cycle have concluded that its role is related both to its activity and its scaffolding effects (Afroze et al., 2014)—this has been demonstrated using two mouse models, one in which there is a complete lack of PMCA4 protein, whilst the other has a mutant, non-functioning PMCA4 (Okunade et al., 2004; Schuh et al., 2004). The availability of the PMCA4 knockout mouse affords the opportunity for investigation of the effects of PMCA4 ablation on BP and arterial contractility. For any clear consensus on the role of PMCA4 in vascular contractility, assessment of a different class of PMCA4 inhibitors, with increased specificity, needs to be made (Pande et al., 2006, 2008; Szewczyk et al., 2008). Indeed, the recent identification of ATA as a highly selective PMCA4 inhibitor (Mohamed et al., 2013) has advanced the pharmacology in this field, and may prove to be a highly useful tool in defining the contribution of PMAC4 to BP regulation.

7. Treatment strategies and future directions

In UK clinical practice it is currently recommended that for people aged 55 or older treatment for hypertension focuses on the use of calcium channel blockers as a first step (NICE, 2011). This highlights the fundamental role Ca^{2+} has for appropriate cardiovascular function. Calcium channel blockers act to inhibit Ca^{2+} influx into cells; Ca^{2+} efflux pathways have not previously been considered for the development of BP lowering pharmacology. The resistance vasculature is an important target for anti-hypertensive therapies and indeed elevated cardiovascular risk remains despite a reduction of BP if inward remodelling of the microcirculation is not reversed (Buus et al., 2013). Calcium channel blockers, and indeed other anti-hypertensive therapies (ACE inhibitors, angiotensin II receptor blockers) have beneficial effects on both BP and small artery structure, others, including diuretics, have little effect on arterial structure (Schiffrin, 2004; Agabiti-Rosei & Rizzoni, 2010). Given the relation to cardiovascular risk, regression of small artery remodelling is an important goal for the development of new anti-hypertensive therapies alongside BP reduction. This may be influenced by changes in resistance arterial contractility (Izzard et al., 1996).

PMCA1 and PMCA4 are the two key PMCAs expressed in cardiovascular tissues. With modulation of both BP and arterial contractility being demonstrated with changes in the expression and/or activity of both PMCA1 (Kobayashi et al., 2012; Shin et al., 2013), and PMCA4 (Gros et al., 2003; Schuh et al., 2003), both have the potential to be new pharmaceutical targets for BP control. The association between *ATP2B1* variants and BP variance and/or hypertension in human subjects (Cho et al., 2009; Levy et al., 2009; Hong et al., 2010; Takeuchi et al., 2010; Johnson et al., 2011) suggests that PMCA1 at least may play an important role in the genetic susceptibility to hypertension. Coupled with the recent observation reporting a genetic link between *ATP2B1* and resistant hypertension (Fontana et al., 2014), there is thus increasing evidence to support PMCA1 as an attractive target for BP and resistance arterial modulation. It is worth noting that whilst effects of PMCA1 on modulation of BP, arterial structure and intracellular calcium have been demonstrated (Kobayashi et al., 2012) there remains a clear need for investigation into the effects of the protein on resistance arterial remodelling. Equally, the effects of PMCA1 on eNOS and on endothelial function need elucidating. Studies to date suggest that PMCA1 expression/activity is inversely correlated with BP suggestive that a specific activator of the pump would need to be developed to reduce BP. Whilst development of these would undoubtedly be challenging, and remains a concept rather than being actively pursued as a therapeutic

strategy, a number of natural PMCA activators have been identified which would help inform work in this area (Lopreiato et al., 2014). PMCA activity has been suggested to be increased by acidic phospholipids (Filomatori & Rega, 2003; Cura et al., 2008). However, PMCAs are not specifically activated by such mechanisms as phosphatidylinositol, and other similar molecules, can also modulate other calcium channels (Kim et al., 2015; Mori et al., 2015). Further, whilst calmodulin binds to an intracellular domain, relieving the autoinhibition on Ca^{2+} flux as a physiological process for PMCA activation (Fig. 2), it too has actions on other channels, negating it as a target for specifically activating PMCA1 (Choi et al., 2014; Alli et al., 2015).

Studies on PMCA4 overexpressing mice which demonstrate an increase in BP (Gros et al., 2003; Schuh et al., 2003) suggest that development of a pharmacological inhibitor of the protein might be an effective anti-hypertensive therapy. However, previous studies using caloxins to inhibit PMCA4 do not support this as such treatment induces an increase in arterial contractility (Pande et al., 2006). Whilst these studies potentially reveal different mechanisms whereby PMCA4 may modulate contractility there is a clear need for assessment of the effects of caloxins on resistance arteries and indeed the effect of more specific inhibitors of PMCA4 on BP and on arterial structure and function. Indeed a more specific inhibitor of PMCA4 has now been identified (Mohamed et al., 2013) which will provide a useful pharmacological tool for further investigating the effects of PMCA4 inhibition on vascular function and on BP. The experimental approaches used to assess the role played by PMCA1 and PMCA4 in BP regulation and in the vasculature, and current knowledge relating to these is shown in Fig. 3. Further molecular understanding of the actions of PMCAs, at a basic science level, is required before new clinical agents may be developed. Use of new pharmacological tools may highlight possible interventional routes, and indeed may be translated into future new antihypertensive therapies.

In summary, whilst strategies to reduce weight and promote a healthy lifestyle do, and will continue to, successfully reduce BP in some patients, new pharmacological therapies are required to treat chronic hypertension which still persists in significant numbers of individuals. Although further work is required to elucidate the precise

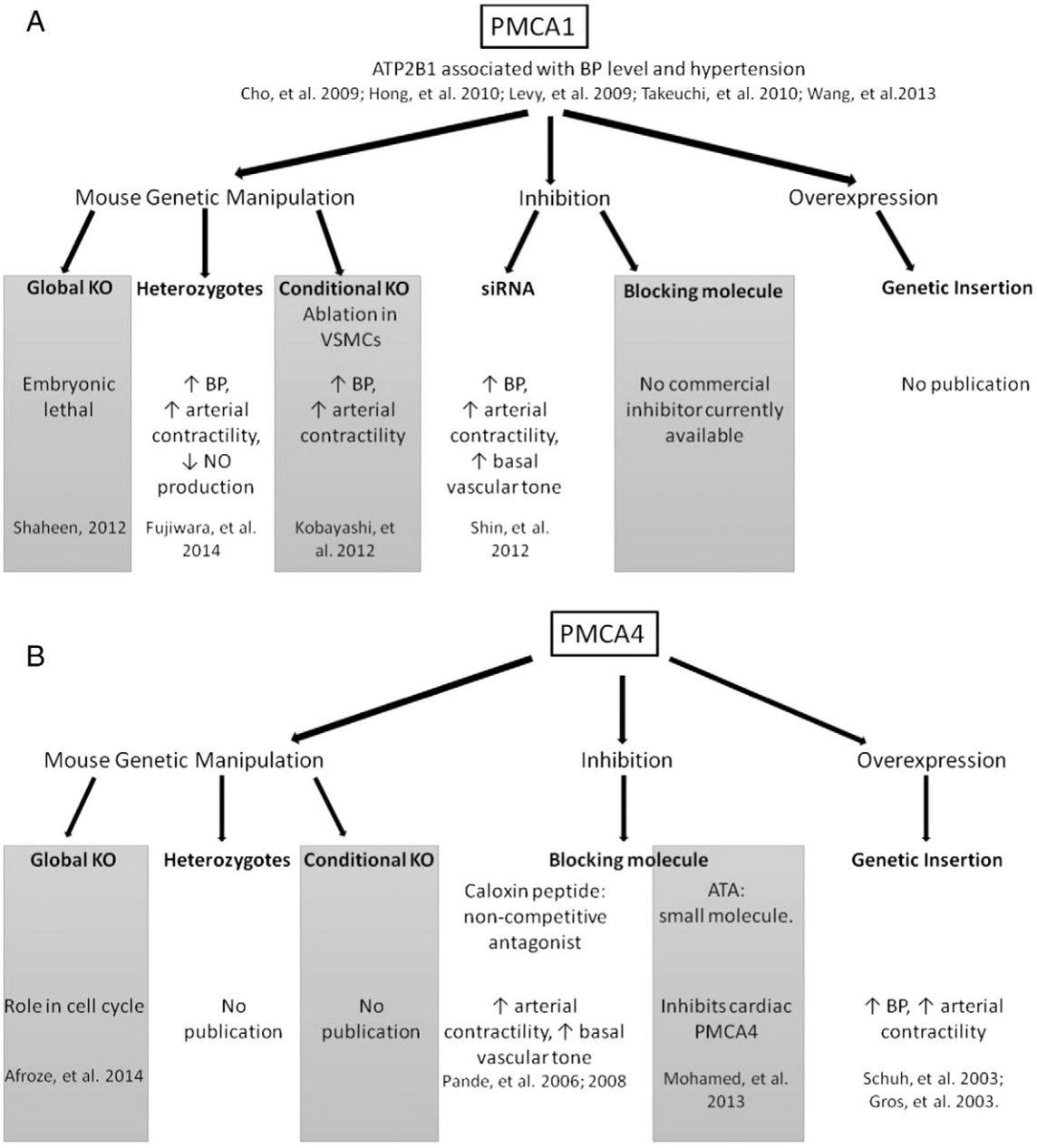

Fig. 3. Outline of experimental approaches assessing how PMCA1 (A.) and PMCA4 (B.) may have a role in vascular function and BP regulation.

role(s) of PMCA1 and 4 in the development and progression of hypertension the clear association between PMCA and BP in humans coupled with experimental evidence supporting a direct role for PMCAs in modulation of arterial function and BP highlights PMCA as a potential new target for the treatment and management of hypertension. This warrants further investigation.

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