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TOPICAL REVIEW

Pivotal role of α2 Na^+ pumps and their high affinity ouabain binding site in cardiovascular health and disease

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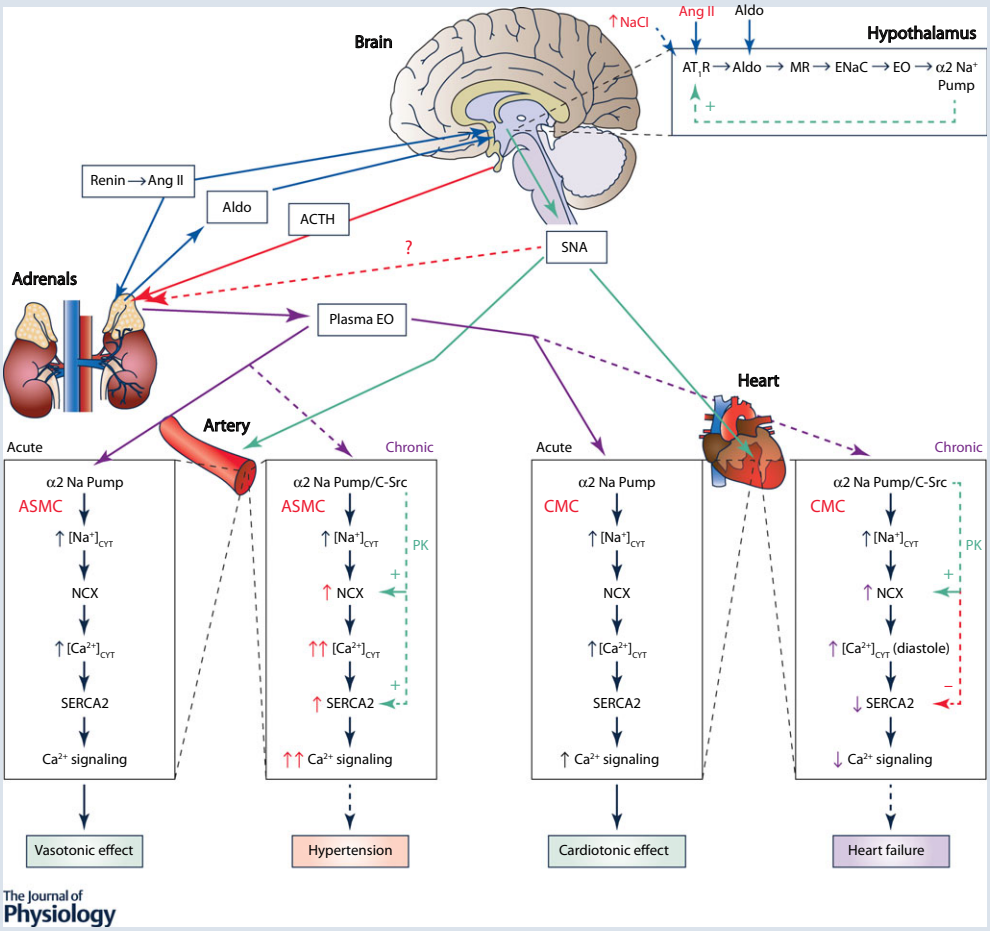

Abstract Reduced smooth muscle (SM)-specific α2 Na^+ pump expression elevates basal blood pressure (BP) and increases BP sensitivity to angiotensin II (Ang II) and dietary NaCl, whilst SM-α2 overexpression lowers basal BP and decreases Ang II/salt sensitivity. Prolonged ouabain infusion induces hypertension in rodents, and ouabain-resistant mutation of the α2 ouabain binding site ($\alpha2^{R/R}$ mice) confers resistance to several forms of hypertension. Pressure overload-induced heart hypertrophy and failure are attenuated in cardio-specific α2 knockout, cardio-specific α2 overexpression and $\alpha2^{R/R}$ mice. We propose a unifying hypothesis that reconciles these apparently disparate findings: brain mechanisms, activated by Ang II and high

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NaCl, regulate sympathetic drive and a novel neurohumoral pathway mediated by both brain and circulating endogenous ouabain (EO). Circulating EO modulates ouabain-sensitive α2 Na^+ pump activity and Ca^{2+} transporter expression and, via Na^+/Ca^{2+} exchange, Ca^{2+} homeostasis. This regulates sensitivity to sympathetic activity, Ca^{2+} signalling and arterial and cardiac contraction.

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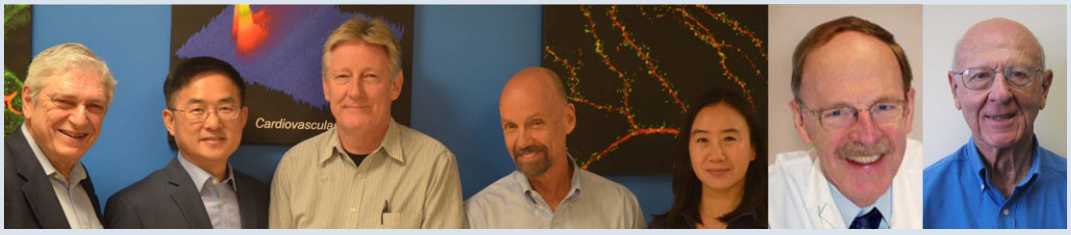
Abstract figure legend The centrally controlled, parallel sympathetic nerve and slow neurohumoral pathways that regulate both arterial and cardiac function and participate in the pathogenesis of hypertension and heart failure (HF). Angiotensin II (Ang II) and high dietary salt are convergent signals that act via hypothalamic Ang type 1 receptors (AT_1R) to activate CNS sympathetic pathways. The increased sympathetic nerve activity (SNA) promotes vasoconstriction and increases cardiac rate and contractile force. Prolonged stimulation of hypothalamic AT_1Rs also activates a novel neurohumoral pathway (box at upper right) that includes aldosterone (Aldo), mineralocorticoid receptors (MR), epithelial Na^+ channels (ENaC), endogenous ouabain (EO) and α2 Na^+ pumps. This hypothalamic pathway feeds back (dashed green line, '+') to modulate Ang II-activated SNA and also promotes adrenal secretion of EO, triggered by, e.g., ACTH, adrenal SNA and/or Ang II. The elevated plasma EO acutely inhibits α2 Na^+ pumps (NKAs) in both the heart and arteries, and the rise in intracellular Na^+ rapidly induces Na^+/Ca^{2+} exchanger (NCX)-mediated Ca^{2+} gain, and cardiotonic and vasotonic effects. Prolonged plasma EO elevation also activates an α2 Na^+ pump-associated protein kinase cascade (e.g. C-Src-mediated) that increases cardiomyocyte (CMC) and arterial smooth muscle cell (ASMC) NCX expression, and arterial sarcoplasmic reticulum (SR) Ca^{2+} pump (SERCA2) expression. In arteries with tone, NCX normally favours Ca^{2+} entry and helps to sustain cytosolic Ca^{2+} ($[Ca^{2+}]_{CYT}$) above contraction threshold. The EO-induced NCX and SERCA2 up-regulation enhance Ca^{2+} signalling and help the very modestly increased SNA to increase vascular tone and resistance, and elevate blood pressure. In the heart, NCX promotes Ca^{2+} extrusion during diastole, but prolonged α2 pump inhibition by EO reduces the Na^+ gradient driving force so that $[Na^+]_{CYT}$ and diastolic $[Ca^{2+}]_{CYT}$ are both elevated; consequently, cardiac relaxation is slow and/or incomplete. Also, cardiac SERCA2 expression is usually reduced in HF (perhaps due to the high EO), as are SR Ca^{2+} stores and Ca^{2+} transients, and systolic function is impaired. The diastolic dysfunction and attenuated cardiac contraction and stroke volume help explain HF. This review describes research on mice with genetically engineered α2 Na^+ pumps and related studies that elucidate these cellular mechanisms.

Abbreviations ACTH, adrenocorticotropic hormone; Ang II, angiotensin II; AT_1R, angiotensin type-1 receptor; BP, blood pressure; CNS, central nervous system; CSF, cerebrospinal fluid; C-Src, C-Src kinase; CTS, cardiotonic steroid; CV, cardiovascular; DN, dominant negative; DOCA, deoxycorticosterone acetate; EDL, extensor digitorum longus; EF, ejection fraction; ENaC, epithelial Na^+ channels; EO, endogenous ouabain; HF, heart failure; HH, heart hypertrophy; I.C.V., intracerebroventricular; jS/ER, junctional sarco-/endoplasmic reticulum; LV, left ventricular; MBG, marinobufagenin; MI, myocardial infarction; MS, mass spectrometry; NCLX, mitochondrial Na^+/Ca^{2+} exchanger; NCX, Na^+/Ca^{2+} exchanger; NKA, Na^+ pump or Na^+, K^+-ATPase; PLM, phospholemman; PM, plasma membrane; RAAS, renin–angiotensin–aldosterone system; ROS, reactive oxygen species; RyR, ryanodine receptor; S.C., subcutaneous; S/ER, sarco-/endoplasmic reticulum; SERCA, sarco-/endoplasmic reticulum Ca^{2+} pump; SkM, skeletal muscle; SM, smooth muscle; SNA, sympathetic nerve activity; SPM, sub-PM; SR, sarcoplasmic reticulum; SS, salt-sensitive; TAC, trans-aortic constriction; TRPC6, transient receptor potential channel-6; WT, wild-type.



Baltimore group (left to right; key mentors in parentheses): **Mordecai Blaustein**, a discoverer of Na^+/Ca^{2+} exchange (NCX), studies arterial Ca^{2+} regulation, arterial tone and hypertension (Daniel Tosteson and Alan Hodgkin). **Ling Chen** investigates cardiovascular mechanisms of hypoxia and ischaema (Morris Karmazyn and Steven Scharf). **John Hamlyn** identified (with Blaustein) endogenous ouabain and its role in hypertension and heart failure (Thomas Duffy and Alan Senior). **Gil Wier**, pioneer of cardiovascular Ca^{2+} signalling *in vitro*, *in situ*, and in awake mice *in vivo* (John Blinks). **Jin Zhang** performed seminal studies on arteries from genetically altered arterial α2 and NCX mice (Xiaoliang Wang and Blaustein). Individual photos. **Frans Leenen** (Ottawa, left) discovered brain ouabain's role in the novel slow neuromodulatory pathway in hypertension and heart failure (Wybren deJong and Alvin Shapiro). **Jerry Lingrel** (Cincinatti), who cloned the Na^+ pump isoforms, studies their physiological roles in genetically engineered mice (Harry Bosook and John Gurdon).

Introduction

A decade ago, an article in this journal (Zhang *et al.* 2005), and two contemporary articles (Dostanic *et al.* 2005; Dostanic-Larson *et al.* 2005), supported the hypothesis (Blaustein, 1977) that arterial Na^+ pumps, their endogenous ouabain-like ligand, and Na^+/Ca^{2+} exchangers (NCX), contribute to salt-sensitive hypertension. The genetically engineered mouse studies implicate the Na^+ pump catalytic subunit α2 isoform. Here, we review recent reports that substantiate the seminal role of α2 Na^+ pumps in the pathogenesis of hypertension and also in cardiac hypertrophy and failure. Remarkably, these pathologies can be prevented/attenuated by genetically altered α2 expression and/or ouabain resistant mutation of its binding site. This pinpoints α2 Na^+ pumps as a key, but largely overlooked, therapeutic target.

Background

Sodium pumps (Na^+,K^+-ATPase or NKA) are expressed in nearly all vertebrate cells. They export three Na^+ ions and import two K^+ ions while hydrolysing one ATP molecule during each transport cycle (Blanco & Mercer, 1998). The Na^+ pumps maintain cell and organism Na^+ and K^+ homeostasis and influence numerous physiological processes. They also serve as cellular signal transducers for cardiotonic steroids (CTSs) (Xie & Askari, 2002).

Four Na^+ pump catalytic subunit isoforms (α1–α4) have been cloned (Shull *et al.* 1985; Shull & Lingrel, 1987; Woo *et al.* 1999). Pumps with an α1 isoform ('α1 Na^+ pumps') are expressed in virtually all cells, and are prevalent in most. They maintain the low Na^+ and high K^+ concentrations in 'bulk' cytoplasm, $[Na^+]_{CYT}$ and $[K^+]_{CYT}$, respectively, and the resting membrane potential (e.g. McDonough *et al.* 1992; He *et al.* 2001; Radzyukevich *et al.* 2013), and mediate net Na^+ transport across epithelia (McDonough *et al.* 1992; Rajasekaran *et al.* 2005). α3 is found in neurones, neonatal myocardium, adult human myocardium, and some other tissues; α4 is expressed in sperm (Lingrel, 2010).

α2 Na^+ pumps. We focus on Na^+ pumps with an α2 catalytic subunit, which are expressed in the cardiovascular (CV) system (Lingrel, 2010), including the endothelium (Zahler *et al.* 1996), in skeletal muscle (Radzyukevich *et al.* 2013) and in the brain (McGrail *et al.* 1991; Arakaki *et al.* 2013). In rodent cardiac and vascular smooth muscles, the α1:α2 ratio is ∼4:1 (James *et al.* 1999; Zhang *et al.* 2005; Berry *et al.* 2007; Despa & Bers, 2007); in skeletal muscle the α1:α2 ratio is ∼1:6 (He *et al.* 2001).

The minimal Na^+ pump functional unit is an αβ protomer (Blanco & Mercer, 1998). The α subunit contains the Na^+, K^+ and Mg-ATP binding sites, the catalytic machinery, and the CTS binding site. Rodents are unusual, however, because their α1 Na^+ pumps have very low affinity for CTS (O'Brien *et al.* 1994). Thus, in rodents, and probably in other orders of mammals too, α2 and α3 Na^+ pumps are the receptors for picomolar to nanomolar CTS (Song *et al.* 2013). CTSs selectively inhibit Na^+ pump-mediated cation transport (Schatzmann, 1953). Therefore, CTSs, and especially ouabain, which is relatively hydrophilic, are widely employed to study the consequences of Na^+ pump blockade.

The Na^+ pump β subunit (there are 3 isosforms) chaperones α, and is essential for the catalytic activity (Shull *et al.* 1986; Blanco & Mercer, 1998; Lingrel, 2010). β1 is the most prevalent isoform in cardiac muscle and vascular smooth muscle, where it forms both α1β1 and α2β1 protomers (Hundal *et al.* 1994; Cougnon *et al.* 2002; Hauck *et al.* 2009; Dey *et al.* 2012).

α2 Na^+ pump localization and its significance. Most arterial (Fig. 1) and cardiac (Fig. 2) myocyte α2 Na^+ pumps are localized in plasma membrane (PM) microdomains in close proximity to 'junctional' elements of the sarco-/endoplasmic reticulum (jS/ER), i.e. at PM–S/ER junctions (Juhaszova & Blaustein, 1997*a*,*b*; Mohler *et al.* 2003; Despa & Bers, 2007; Linde *et al.* 2012). There may, however, be some overlap with α1 in these microdomains (Mohler *et al.* 2003; Dey *et al.* 2012). Na^+/Ca^{2+} exchangers (NCX), too, are localized in the PM–jS/ER microdomains (Figs 1 and 2) (Juhaszova & Blaustein, 1997*a*; Berry *et al.* 2007; Lynch *et al.* 2008; Davis *et al.* 2009; Jayasinghe *et al.* 2009; Kuszczak *et al.* 2010). In cardiomyocytes, α2 pumps and NCX are found at, or adjacent to (Scriven *et al.* 2000), PM–S/ER junctions in the transverse (t-) tubules as well as in the surface membrane (Fig. 2) (Mohler *et al.* 2003; Berry *et al.* 2007; Despa & Bers, 2007).

This organization enables privileged communication among the α2 Na^+ pumps, NCX and S/ER Ca^{2+} pumps (SERCA) through the tiny sub-PM cytosolic compartment, 'fuzzy space', at the junctions (Figs 3 and 4) (Juhaszova & Blaustein, 1997*b*; Goldhaber *et al.* 1999; Poburko *et al.* 2004; Verdonck *et al.* 2004; Pritchard *et al.* 2010; Swift *et al.* 2010; Aronsen *et al.* 2013). Consequently, the *local* α2 pump-generated Na^+ electrochemical gradient (Poburko *et al.* 2007) modulates NCX-mediated Ca^{2+} transport, and local Ca^{2+} sequestration and Ca^{2+} signalling (Blaustein & Lederer, 1999; Arnon *et al.* 2000*b*; Golovina *et al.* 2003; Verdonck *et al.* 2004; Lee *et al.* 2006; Lingrel, 2010; Despa *et al.* 2012; Shattock *et al.* 2015). This α2-NCX linkage is consistent with the observation that ∼75% knockdown of cardiac NCX1 decreased α2, but not α1, expression by ∼50% (Bai *et al.* 2013). Also, ∼65% knockdown of α2 decreased NCX1 by ∼65%, but did not affect α1 expression in arteries (Chen *et al.* 2015*b*).

The special role of α2 also is evident in skeletal muscle: α2 Na^+ pumps and NCX are prevalent in t-tubules, where they may coordinate with junctional sarcoplasmic

reticulum (SR) to help regulate the SR Ca^{2+} concentration, $[Ca^{2+}]_{SR}$, and contraction (Radzyukevich *et al.* 2013; Altamirano *et al.* 2014; DiFranco *et al.* 2015). Skeletal myocytes (Kristensen *et al.* 2008) (and choroid epithelial cells; Arakaki *et al.* 2013), are unusual in that most $\alpha 2$ Na^+ pumps are located in intracellular vesicles and are inactive. When translocated to the PM, triggered, e.g., by insulin, muscle contraction or cyclic stretching, they become active (Therien & Blostein, 2000; Yuan *et al.* 2007; Benziane & Chibalin, 2008; Kristensen *et al.* 2008; Zhang *et al.* 2012).

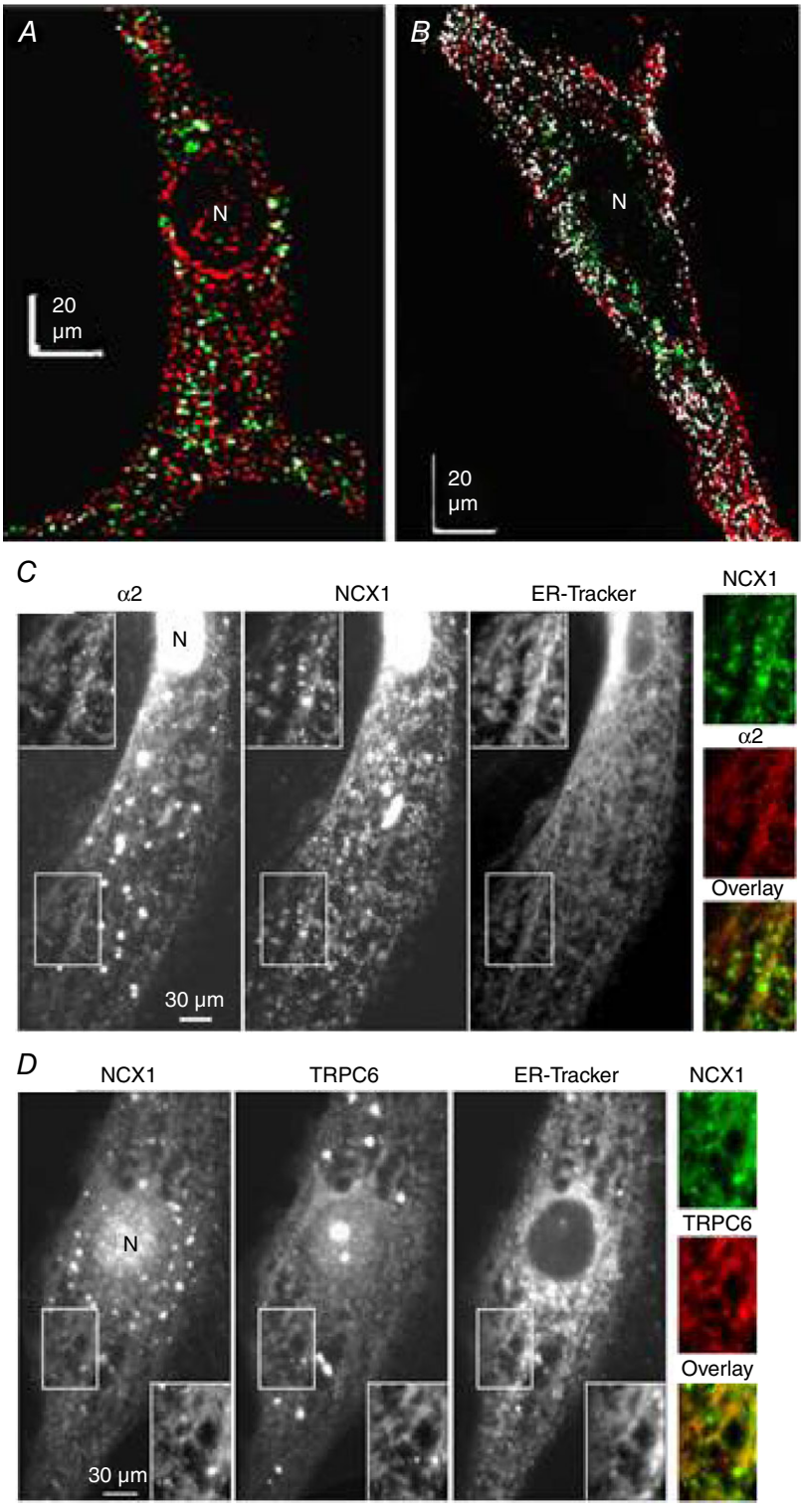


Figure 1. Distribution of $\alpha 2$ Na^+ pumps and NCX1 in embryonic mouse (*A* and *B*) and human (*C* and *D*) artery smooth muscle determined by immunocytochemistry
A, $\alpha 2$ Na^+ pumps (green) and the plasma membrane Ca^{2+} pump (PMCA, red) are not co-localized (negligible white areas) in this pseudocolour overlay image of a mouse aorta myocyte. *B*, $\alpha 2$ Na^+ pumps (green) and NCX1 (red) exhibit substantial co-localization (white areas) in a mouse aorta myocyte. *C*, primary cultured human mesenteric artery smooth muscle cells (hASMCs) were labelled with anti-$\alpha 2$ polyclonal antibodies (pAb) and anti-NCX1 monoclonal antibodies (mAb); the SR was then stained with ER-Tracker, as indicated by the labels. Insets are enlargements of the boxed areas. Pseudocolour images of the enlarged $\alpha 2$ (red) and NCX1 (green) regions, and the overlay, are shown on the right. *D*, hASMCs were cross-reacted with anti-NCX1 mAb and anti-TRPC6 pAb; the SR was then stained with ER-Tracker, as indicated. Insets are enlargements of the boxed areas. Pseudocolour images of the enlarged NCX1 (green) and TRPC6 (red) regions, and the overlay, are shown on the right. In *C* and *D*, the patterns of staining by both antibodies were very similar to the pattern of ER Tracker (i.e. SR) distribution. Scale bars in *C* and *D* = 30 μm. Note that the $\alpha 2$, NCX1 and TRPC6 staining patterns are all very similar to that of ER-Tracker. This is reflected by the yellow-orange staining in the *C* and *D* overlay panels, and indicates that hASMC $\alpha 2$ Na^+ pumps and NCX1 co-localize (as in the mouse, *B*) and overlie elements of SR. *A* and *B* were kindly provided by Dr Ronald P. Lynch (*B* is from Lynch *et al.* 2008 with permission); *C* and *D* are from Linde *et al.* (2012) with permission.

Regulation of α2 Na^+ pumps; role of phospholemman. Na^+ pumps, including α2 pumps, are regulated by multiple factors, including substrates, hormones (e.g. aldosterone, insulin and catecholamines) and protein phosphorylation (Therien & Blostein, 2000; Phakdeekitcharoen *et al.* 2011). Importantly, the Na^+ and K^+ affinities are modulated by the regulatory protein phospholemman (PLM), also called FXYD1 (Crambert *et al.* 2002; Bibert *et al.* 2008; Bossuyt *et al.* 2009; Han *et al.* 2010; Mishra *et al.* 2015). Surprisingly, this small molecule with a single transmembrane helix (Geering, 2006) also regulates NCX1 (Wang *et al.* 2011; Hafver *et al.* 2016).

Unphosphorylated PLM binds to $\alpha 2\beta$ and reduces α2 affinity for intracellular Na^+ and extracellular K^+ (Han *et al.* 2009; Pavlovic *et al.* 2013*a*). Phosphorylation of cardiac or arterial PLM by protein kinase A or C relieves the pump inhibition by altering PLM-$\alpha 2\beta$1 interaction and restoring the Na^+ high affinity (Bossuyt *et al.* 2006, 2009; Pavlovic *et al.* 2007, 2013*a*; Han *et al.* 2010; Dey *et al.* 2012; Shattock *et al.* 2015).

Activation of the renin–angiotensin (Ang)–aldosterone system (RAAS), as in hypertension and heart failure (see below), stimulates reactive oxygen species (ROS) generation. This leads to glutathionylation of β1 and pump inhibition (Figtree *et al.* 2009; Liu *et al.* 2013). PLM promotes de-glutathionylation and protects against oxidative inhibition of the pumps in arteries and heart (Liu *et al.* 2013; Chia *et al.* 2015).

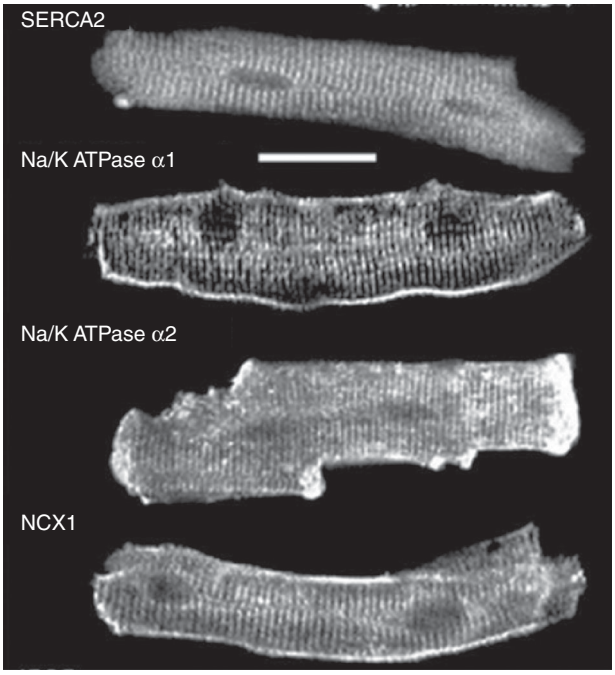


Figure 2. Confocal images of normal adult rat cardiomyocytes immunolabelled with antibodies raised against SERCA2, Na^+ pump α1, Na^+ pump α2 and NCX1
All four antibodies stained the Z-line/t-tubule regions. The surface membrane was stained by anti-α1, anti-NCX1 and, to a much lesser extent, anti-α2 antibodies, but not by anti-SERCA2. Scale bar = 40 μm. Reproduced from Mohler *et al.* (2003) with permission.

Endogenous cardiotonic steroids. High affinity CTS binding is observed in all vertebrates (Pressley, 1992; Lingrel, 2010). Ouabain, digoxin and bufalin (a bufadienolide CTS) all block the pump's cation transport pathway (Laursen *et al.* 2013, 2015). The idea of an endogenous ligand for the Na^+ pump CTS binding site (Szent-Gyorgi, 1953) fostered the proposal that an endogenous ouabain-like compound contributes to the pathogenesis of hypertension (Haddy & Overbeck, 1976; Blaustein, 1977). Studies in mice with an α2 null mutation, $\alpha 2^{+/-}$ and, especially, with ouabain-resistant α2 Na^+ pumps, $\alpha 2^{R/R}$, provide definitive evidence that α2 and its endogenous ligand(s) have a physiological role in mammals (Dostanic *et al.* 2005; Dostanic-Larson *et al.* 2005; Zhang *et al.* 2005; Lingrel, 2010; Van Huysse *et al.* 2011; Wansapura *et al.* 2011).

A CTS was isolated from human plasma and was identified by mass spectrometry (MS) as endogenous ouabain (EO) or a ouabain isomer (Hamlyn *et al.* 1991; Mathews *et al.* 1991). Nuclear magnetic resonance (NMR) and MS studies on human, bovine and rodent plasma and tissues verified that the endogenous substance is ouabain (Schneider *et al.* 1998; Kawamura *et al.* 1999; Komiyama *et al.* 2000; Tashko *et al.* 2010; Jacobs *et al.* 2012; Hamlyn *et al.* 2014); reviewed in Hamlyn & Blaustein, 2016). Moreover, recent studies identified two novel EO isomers that are not seen in commercial (plant) ouabain (Jacobs *et al.* 2012; Hamlyn *et al.* 2014). The isomers, which may also be present in human plasma (Hamlyn & Blaustein, 2016), apparently are physiologically regulated, but their relative affinities for α2 and their significance are unknown.

Another CTS, marinobufagenin (MBG), was identified in human plasma and urine by immunoassay (Bagrov *et al.* 1996, 2009). Both EO and MBG reportedly play a role in the pathogenesis of hypertension and heart failure (HF) (Schoner & Scheiner-Bobis, 2007; Bagrov *et al.* 2009; Blaustein *et al.* 2012; Pavlovic, 2014). Prolonged administration of ouabain (Doursout *et al.* 1992; Yuan *et al.* 1993; Huang *et al.* 1994) or MBG (Kennedy *et al.* 2006) induces hypertension in normal rats, but digoxin and digitoxin do not (Manunta *et al.* 2000). This implies that Na^+ pump inhibition *per se* does not cause the ouabain- or MBG-induced hypertension. Experiments on $\alpha 2^{R/R}$ mice (see Table 1), and studies with fab fragments that immunoneutralize EO and MBG, demonstrate that elevated endogenous CTS levels contribute to CV pathophysiology (Schoner & Scheiner-Bobis, 2007; Bagrov *et al.* 2009; Blaustein & Hamlyn, 2010). This review focuses on EO and not

Table 1. Cardiovascular and skeletal muscle manifestations of genetically modified mouse α2 Na^+ pumps

Genetic modification	Vascular effects	Cardiac effects	Skeletal muscle effects	References
Globally reduced α2 ($\alpha2^{+/-}$)	↑Basal $[Ca^{2+}]_{CYT}$; ↑myogenic tone; ↑basal BP; I.C.V. Ang I and ↑$[Na^+]_{CSF}$ induce hypertension	↑$[Ca^{2+}]_{CYT}$ transients; ↑contractility	↑Contractile force	(James *et al.* 1999) (He *et al.* 2001) (Shelly *et al.* 2004) (Zhang *et al.* 2005) (Hou *et al.* 2009)
Smooth muscle α2 dominant negative ($\alpha2^{SM\text{-}DN}$)	↑Basal BP; ↓myogenic reactivity; ↑pressor response to Ang II + high dietary salt	—	—	(Chen *et al.* 2015*b*)
Smooth muscle α2 transgenic over-expressor (SM-$\alpha2^{Tg/Tg}$)	↓Basal BP; normal myogenic reactivity; ↓pressor response to Ang II ± high dietary salt	—	—	(Pritchard *et al.* 2007) (Chen *et al.* 2015*b*)
CV α2 null (CV-$\alpha2^{-/-}$)	Normal basal BP; No ACTH- or ouabain-induced hypertension	Normal basal cardiac function	—	(Rindler *et al.* 2011)
Cardiac α2 null (Cardiac-$\alpha2^{-/-}$)	Normal basal BP; ACTH induces hypertension	Normal basal cardiac function; attenuated TAC-induced cardiac hypertophy	—	(Rindler *et al.* 2013)
Cardiac α2 (Tg) transgenic over-expressor (Cardiac-$\alpha2^{Tg}$)	—	Attenuated TAC-induced cardiac hypertophy	—	(Correll *et al.* 2014)
Cardiac α1 (Tg) transgenic over-expressor (Cardiac-$\alpha1^{Tg}$)	—	TAC-induced cardiac hypertophy	—	(Correll *et al.* 2014)
Skeletal muscle α2 null ($\alpha2^{SkM-/-}$)	—	—	↓Contractile force; ↑sensitivity to fatigue	(Radzyukevich *et al.* 2009) (Radzyukevich *et al.* 2013)
Global ouabain-resistant α2 ($\alpha2^{R/R} = \alpha1^{R/R}\text{-}\alpha2^{R/R}$)	Normal basal BP; no ACTH- or ouabain-induced hypertension; no I.C.V. ouabain- or ↑$[Na^+]_{CSF}$-induced hypertension; ↓BP in 3rd trimester of pregnancy; DOCA + salt induces hypertension	No CTS-induced cardiotonic effect; attenuated TAC-induced cardiac hypertophy	↓Sensitivity to fatigue	(Dostanic *et al.* 2003) (Dostanic *et al.* 2005) (Dostanic-Larson *et al.* 2005) (Lorenz *et al.* 2008) (Radzyukevich *et al.* 2009) (Oshiro *et al.* 2010) (Van Huysse *et al.* 2011) (Despa *et al.* 2012) (Lorenz *et al.* 2012)
Global SWAP ($\alpha1^{S/S}\text{-}\alpha2^{R/R}$ *vs.* WT $= \alpha1^{R/R}\text{-}\alpha2^{S/S}$)	—	↓Contractility; ↓sensitivity to CTS-induced cardiotonic effect (*vs.*WT); accelerated TAC-induced cardiac hypertrophy	—	(Dostanic *et al.* 2004) (Wansapura *et al.* 2009) (Wansapura *et al.* 2011) (Despa *et al.* 2012)

MBG because: (i) DigiBind and DigiFab, commercial fab fragments used to immunoneutralize endogenous CTS *in vivo*, bind ouabain with much higher affinity than MBG (Pullen *et al.* 2004, 2008); (ii) MBG preferentially binds to α1 rather than α2 subunits (Wansapura *et al.* 2009); and (iii) several clinical and animal studies on the functions of EO in CV physiology and pathophysiology are backed by analytical (MS) measurements, e.g. Stella *et al.* (2008), Jacobs *et al.* (2012) and Hamlyn *et al.* (2014).

Ouabain-triggered, Na^+ transport-independent cell signalling mediated by Na^+ pumps. Prolonged treatment with ouabain activates multiple intracellular signalling pathways independent of effects on Na^+ transport in

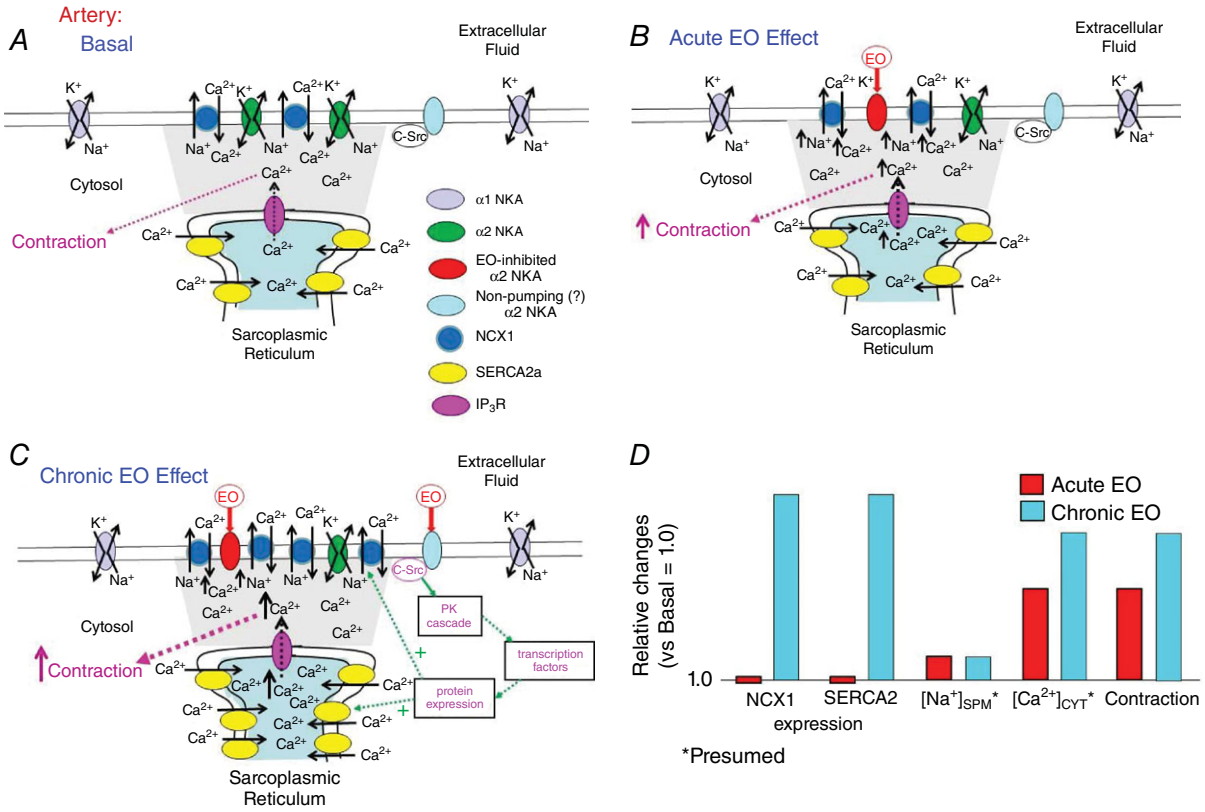


Figure 3. Diagrams illustrating the acute and chronic effects of EO on Ca^{2+} homeostasis in arteries: roles of α2 Na^+ pumps (NKA), NCX1, SERCA2 and inositol trisphosphate receptors (IP_3R)
Other Ca^{2+} transporters such as L-type voltage-gated Ca^{2+} channels and plasma membrane (PM) Ca^{2+} pumps (PMCA) are omitted for simplicity. *A*, basal conditions. In arteries with tone, myocyte NCX1 operates primarily in the Ca^{2+} entry mode because the membrane potential, $V_m = -35$ to -50 mV, is more positive than the NCX1 'reversal potential', $E_{Na/Ca}$ (Blaustein & Lederer, 1999); i.e. the driving force ($V_m - E_{Na/Ca}$) is positive. *B*, acute exposure of arteries to low dose ouabain or EO inhibits (a fraction of) arterial myocyte α2 Na^+ pumps, raises $[Na^+]$ in the sub-PM restricted cytosolic space between the PM and SR (shaded area; i.e. $[Na^+]_{SPM}$)*, thereby increasing $E_{Na/Ca}$ and the driving force for NCX1-mediated Ca^{2+} entry. The consequent rise in $[Ca^{2+}]_{CYT}$ and Ca^{2+} sequestered in the SR augments Ca^{2+} signalling and contraction (the vasotonic effect), thereby increasing vascular tone and BP. *C*, sustained exposure of arterial myocytes to low dose ouabain or EO, in addition to its acute effects, activates an α2 Na^+ pump-mediated protein kinase (PK) signalling cascade that leads to increased expression of Ca^{2+} transporters including NCX1 and SERCA (green dotted line and '+' sign). This promotes long-term arterial Ca^{2+} gain and sequestration in the SR; via increased Ca^{2+} signalling, this leads to long-term elevation of BP. *D*, comparison of approximate acute and chronic EO-induced relative changes in NCX1 and SERCA2 expression and contraction, and anticipated $[Na^+]_{SPM}$* and $[Ca^{2+}]_{CYT}$*. The α2 Na^+ pump–NCX1 functional coupling acts as an amplifier: small increases in $[Na^+]_{SPM}$ translate to large increases in $[Ca^{2+}]_{CYT}$ and contraction because of the 3 Na^+:1 Ca^{2+} stoichiometry of NCX1 (Blaustein & Lederer, 1999). Furthermore, arterial resistance is inversely related to the fourth power of the radius, r^4 (Poiseuille's law), so small decreases in the radii of resistance arteries will greatly increase peripheral vascular resistance and BP. *Note: $[Na^+]_{SPM}$ has not been measured in arterial myocytes, nor have the acute and chronic effects of EO/ouabain on $[Ca^{2+}]_{CYT}$ been compared; thus, the relative changes shown in the figure are speculative. The anticipated $[Na^+]_{SPM}$ changes are consistent with NCX1-mediated Ca^{2+} entry during chronic high EO (Iwamoto *et al.* 2004) and with the evidence that immuno-neutralization of EO rapidly decreases BP in mice with chronic Ang II + salt-induced hypertension (Chen *et al.* 2015*a*).

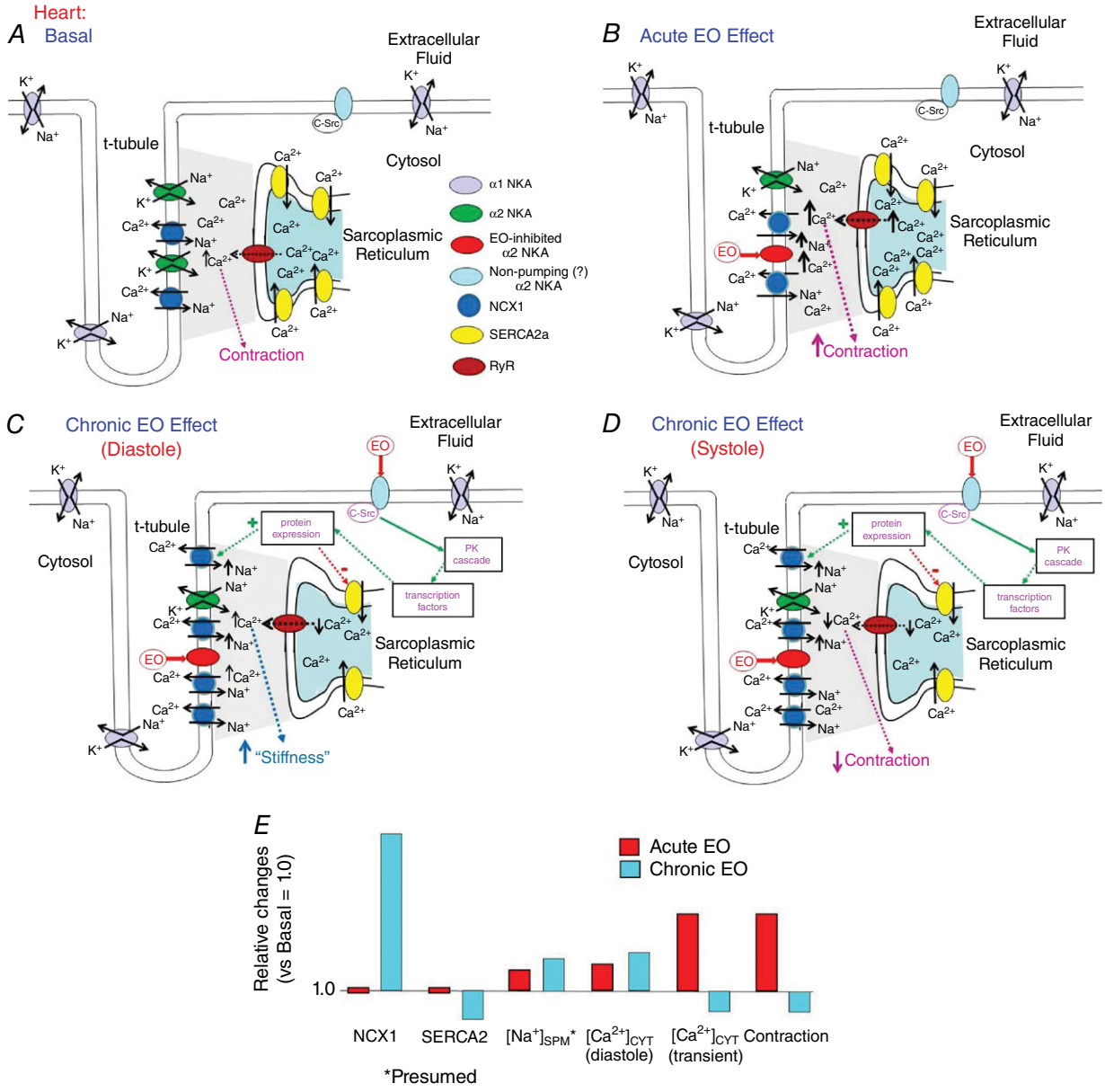


Figure 4. Diagrams illustrating the acute and chronic effects of EO on Ca^{2+} homeostasis in the heart: roles of α2 Na^+ pumps (NKA), NCX1, SERCA2 and ryanodine receptors (RyR)
A, basal conditions. In cardiac myocytes, during the major part of the cardiac cycle the NCX1 operates in the Ca^{2+} exit mode because the diastolic V_m, perhaps about −65 to −75 mV, is more negative than $E_{Na/Ca}$; i.e. the driving force ($V_m - E_{Na/Ca}$) is negative. *B*, acute exposure of the heart to low dose ouabain or EO inhibits cardiac myocyte α2 Na^+ pumps and raises $[Na^+]_{SPM}$. This increases $E_{Na/Ca}$, but reduces the driving force for Ca^{2+} extrusion and elevates $[Ca^{2+}]_{SPM}$. Thus, the net effect, as in arteries, is enhanced Ca^{2+} signalling and contraction (i.e. the cardiotonic effect). *C* and *D*, sustained exposure of cardiac myocytes to low dose ouabain or EO also, as in arteries, activates an α2 Na^+ pump-mediated protein kinase (PK) signalling cascade. In the heart, however, this leads to increased NCX1 expression, but decreased SERCA expression (green and red dotted lines and '+' and '−', respectively). Thus, initially, the cytosolic and SR $[Ca^{2+}]$ are elevated, the cardiotonic effect prevails, increased cardiac contraction is sustained (as in '*B*'), and the heart may hypertrophy from the increased workload. Eventually, however, the sustained Na^+ pump inhibition and $[Na^+]_{CYT}/[Na^+]_{SPM}$ elevation will maintain an elevated diastolic $[Ca^{2+}]_{CYT}$ (*C*) despite the up-regulated NCX1. The decreased SERCA2 expression and leakage of Ca^{2+} from the SR via RyR, however, reduces SR Ca^{2+} sequestration and $[Ca^{2+}]_{CYT}$ transients (*D*); thus, cardiac contraction decreases, and the heart fails. *E*, summary of the acute and chronic EO-induced approximate relative changes in NCX1 and SERCA2 expression, $[Na^+]_{SPM}$ (postulated; see Fig. 3 legend), $[Ca^{2+}]_{CYT}$ and contraction. Note that the acute vasotonic (Fig. 3*B* and *D*) and cardiotonic effects of EO are similar, whereas the chronic effects of EO in the heart (*C*–*E*) differ greatly from those in arteries (Fig. 3*C*, *D*).

rat heart and other tissues (Xie & Askari, 2002; Tian & Xie, 2008; Li & Xie, 2009; Zulian *et al.* 2013). The ouabain-activated signalling may be mediated by a separate, 'non-pumping pool' of pumps (Liang *et al.* 2007), perhaps located in caveolae (Liu *et al.* 2003; Wang *et al.* 2004; Kristensen *et al.* 2008). Most investigations employed 1–100 μM ouabain, and emphasized the participation of rodent $\alpha1$ Na^+ pumps, which are relatively ouabain resistant (Liang *et al.* 2006; Tian *et al.* 2006). The effectiveness of submicromolar ouabain in rat tissues (Liu *et al.* 2000; Pulina *et al.* 2010; Zulian *et al.* 2013), however, implies mediation by ouabain-sensitive $\alpha2$ (or $\alpha3$), and not resistant $\alpha1$ Na^+ pumps. Ouabain-induced cell signalling was not observed in immortalized $\alpha1$-deficient cells transfected with rat $\alpha2$ (Xie *et al.* 2015), but there is no evidence that $\alpha2$ was linked to the appropriate signalling molecules in those cells, which do not normally express $\alpha2$. This requires direct comparison of low dose ouabain in native cells from wild-type (WT) and $\alpha2^{R/R}$ mice.

Ouabain-triggered intracellular signalling involves protein kinase cascades such as C-Src kinase (C-Src), ERK1/2, MAPK, phosphatidylinositide 3-kinase 1A, protein kinase B (Akt) and NF-κB, and may be cell-type specific (Xie & Askari, 2002; Li & Xie, 2009; Wu *et al.* 2013). C-Src can be activated by ouabain-induced ROS generation and carbonylation of the pump (Yan *et al.* 2013).

Functions of α2 Na+ pumps in arterial physiology and pathophysiology

Arterial myocyte α2 Na+ pumps, modulation of vasoconstriction and blood pressure. Most, if not all, $\alpha2$ pumps in vascular smooth muscle co-localize with NCX1 at the PM–jS/ER where, together, they help regulate Ca^{2+} homeostasis and influence Ca^{2+} signalling and vasoconstriction (Juhaszova & Blaustein, 1997*a*; Lynch *et al.* 2008; Linde *et al.* 2012). Normally, most arteries maintain myogenic or vasoconstrictor-induced (mainly sympathetic nerve-mediated) 'basal' tone (Hill *et al.* 2001; Zhang *et al.* 2010*a*). Myocyte membrane potential is in the order of −35 to −50 mV (Knot & Nelson, 1998), and the electrochemical driving force on NCX (Blaustein & Lederer, 1999) favours net Ca^{2+} entry (Iwamoto *et al.* 2004; Zhang *et al.* 2010*b*; Wang *et al.* 2015) (Fig. 3*A*). Consequently, reduced $\alpha2$ Na^+ pump activity (e.g. ouabain inhibition or reduced expression) should raise the local, sub-PM Na^+ concentration ($[Na^+]_{SPM}$) and promote net Ca^{2+} gain via NCX, thereby enhancing Ca^{2+} stores and signalling, and increasing vascular tone and BP (Fig. 3*B*) (Zhang *et al.* 2005, 2009; Chen *et al.* 2015*b*).

In fact, ouabain induces hypertension in most strains of rats; the few negative reports (Ghadhanfar *et al.* 2014) are consistent with the evidence that ouabain sensitivity is genetically controlled (Aileru *et al.* 2001). Ouabain also induces hypertension in WT, but not $\alpha2^{R/R}$ mice (Dostanic *et al.* 2005). Further, $\alpha2^{R/R}$ mice are resistant to adrenocorticotropic hormone (ACTH)-induced hypertension, and ACTH hypertension is prevented by DigiBind and by the NCX antagonist KB-R7942 (Dostanic-Larson *et al.* 2005; Lorenz *et al.* 2008). Mice in which CV $\alpha2$ pumps are selectively knocked out (CV-$\alpha2^{-/-}$ mice) are also resistant to ACTH-induced hypertension (Rindler *et al.* 2011). Because ACTH stimulates EO secretion (Laredo *et al.* 1994), the implication is that EO-induced inhibition of $\alpha2$ raises $[Na^+]_{SPM}$ and promotes NCX1-mediated net gain of Ca^{2+} and increased arterial constriction.

Mice with genetically reduced $\alpha2$ pump expression, whether global $\alpha2$ heterozygous null mutants, $\alpha2^{+/-}$ (Zhang *et al.* 2005), or smooth muscle (SM)-specific dominant negative (DN) knockdown, $\alpha2^{SM\text{-}DN}$ (Chen *et al.* 2015*b*), also have elevated BP. (Global $\alpha2^{-/-}$ is embryonic lethal; James *et al.* 1999.) The $\alpha2^{SM\text{-}DN}$ mice have increased BP sensitivity to subcutaneous (S.C.) Ang II and high dietary salt (*vs.* WT; not tested in $\alpha2^{+/-}$ mice), presumably because there are fewer available $\alpha2$ EO receptors and a larger fraction are inhibited by the elevated EO (Blaustein *et al.* 2015; Chen *et al.* 2015*b*). Conversely, mice with SM-specific $\alpha2$ overexpression ($\alpha2^{SM\text{-}Tg}$) and excess $\alpha2$ EO binding sites, have low basal BP (Pritchard *et al.* 2007; Chen *et al.* 2015*b*) and reduced BP sensitivity to S.C. Ang II and high dietary salt (Chen *et al.* 2015*b*).

The fact that CV-$\alpha2^{-/-}$ mice have normal basal BP despite the nearly complete absence of arterial SM $\alpha2$ Na^+ pumps (Rindler *et al.* 2011) seems inconsistent with these other reports. In CV-$\alpha2^{-/-}$ mice, however, the $\alpha2$-NCX1 coupling at PM–S/ER junctions is disrupted and NCX1-mediated Ca^{2+} transport is stabilized by overexpression of $\alpha1$ Na^+ pumps (Rindler *et al.* 2011) which maintain a constant, low global $[Na^+]_{CYT}$ and are resistant to ouabain/EO.

Digibind lowers BP in deoxycorticosterone acetate (DOCA)–salt hypertensive rats (Krep *et al.* 1995), and their arteries overexpress the Ca^{2+} transporter transient receptor potential channel-6 (TRPC6) (NCX1 and SERCA2 were not tested) (Bae *et al.* 2007), suggesting that EO is involved. However, $\alpha2^{R/R}$ mice develop DOCA–salt hypertension (Lorenz *et al.* 2012), implying that EO and MBG are not involved. Whether this is a species or technical difference is unknown.

Collectively, the above reports demonstrate that arterial SM $\alpha2$ Na^+ pumps and EO, along with arterial NCX1, modulate arterial tone and BP, and play an important role in some forms of hypertension (Table 1 and Fig. 3).

Brain α2 Na+ pumps and hypertension. The role of the central nervous system (CNS) in the pathogenesis of essential hypertension and salt-sensitive (SS-) hypertension is well documented, albeit incompletely

understood. There is broad agreement that central sympathetic drive is a major contributor to BP elevation (Fisher & Fadel, 2010; Allen, 2011; Gabor & Leenen, 2012; Stocker *et al.* 2015). In addition, EO in the hypothalamus ('brain ouabain') and brain $\alpha2$ Na^+ pumps play a role in the pathogenesis of rodent SS-hypertension: In Wistar rats (Leenen, 2010; Gabor & Leenen, 2012) and WT mice, but not in $\alpha2^{R/R}$ mice (Van Huysse *et al.* 2011), prolonged intracerebroventricular (I.C.V.) infusion of Na^+-rich cerebrospinal fluid (CSF) or very low dose ouabain elevates BP. These effects are augmented in $\alpha2^{+/-}$ mice (Hou *et al.* 2009), presumably because there are fewer available $\alpha2$ EO receptors and a larger fraction are inhibited. Further, I.C.V. infusion of anti-ouabain, but not control, fab fragments prevents the Na^+-rich CSF-induced BP elevation (Huang *et al.* 2006; Van Huysse *et al.* 2011). Clearly, $\alpha2$ Na^+ pumps, their CTS binding site, and the endogenous ligand are all critical for SS-hypertension.

Salt-sensitive hypertension is also attenuated by I.C.V. infusion of the epithelial Na^+ channel (ENaC) inhibitor benzamil (Gomez-Sanchez *et al.* 1996; Leenen, 2010; Gabor & Leenen, 2012; Van Huysse *et al.* 2012; Osborn *et al.* 2014). Brain ENaCs are expressed in neurones and glia, and in the choroid plexus and ependyma (Amin *et al.* 2005; Leenen, 2010; Miller & Loewy, 2013; Miller *et al.* 2013; Oshima *et al.* 2013). Knockout of the ubiquitin ligase Nedd4-2, a regulator of ENaC expression, enhances ENaC activity in the kidney and brain, and Nedd4-$2^{-/-}$ mice develop moderate SS-hypertension (Shi *et al.* 2008; Van Huysse *et al.* 2012). When crossed with $\alpha2^{R/R}$ mice, the double mutants (Nedd4-$2^{-/-}$-$\alpha2^{R/R}$ mice) had a markedly attenuated BP elevation, compared to Nedd4-$2^{-/-}$ mice, in response to either Na^+-rich CSF (I.C.V.) or high dietary salt (Leenen *et al.* 2015). Thus, both arterial and brain $\alpha2$ Na^+ pumps, and their endogenous ligand, contribute to SS-hypertension. The locus of the relevant brain $\alpha2$ pumps is unknown, but $\alpha2$ is expressed in meningeal capillary endothelia, in the choroid epithelial cell cytoplasm (Arakaki *et al.* 2013), and in neurones and glia (McGrail *et al.* 1991; Moseley *et al.* 2003). The cytoplasmic pumps may be cycled to the PM (Benziane & Chibalin, 2008) under conditions yet to be determined.

Ouabain-triggered, α2-mediated cell signalling and hypertension. Prolonged exposure to nanomolar ouabain increases expression of several arterial Ca^{2+} transporters, both in rats *in vivo* (i.e. during hypertension induction), and in primary cultured human and rat artery myocytes. Ca^{2+} signalling is then augmented even after ouabain washout (Pulina *et al.* 2010; Linde *et al.* 2012; Zulian *et al.* 2013). The same proteins, most notably NCX1 and SERCA2, are also up-regulated in several rodent hypertension models, including Dahl-salt-sensitive, Milan, and spontaneously hypertensive rats, and the Ang II, Ang II + salt and DOCA + salt models (Blaustein *et al.* 2012, 2015; Pulina *et al.* 2013). This is consistent with the idea that circulating EO is elevated in these models and that it initiates these changes in protein expression. Arterial NCX1 is also up-regulated in human primary pulmonary hypertension (Zhang *et al.* 2007). Prolonged ouabain/EO–$\alpha2$ interaction triggers arterial myocyte C-Src phosphorylation, reduces ERK1/2 phosphorylation, and leads to the Ca^{2+} transporter reprogramming (Fig. 3*C*) (Zulian *et al.* 2013). Importantly, both the acute and chronic actions of EO and ouabain augment arterial Ca^{2+} entry and signalling. Both should therefore foster vasoconstriction and BP elevation in essential hypertension, primary aldosteronism, and other forms of hypertension in which plasma EO is elevated (Fig. 3*B–D*) (Blaustein & Hamlyn, 2010; Blaustein *et al.* 2012).

CTS–$\alpha2$ Na^+ pump interactions are more complex than anticipated. All CTSs inhibit $\alpha2$ (and $\alpha3$) Na^+ pumps, augment Ca^{2+} signalling (Song *et al.* 2013) and have similar acute vasotonic effects (Song *et al.* 2014). In contrast, ouabain-like CTSs (e.g. *Strophanthus* steroids), but not digoxin-like CTSs (e.g. *Digitalis* steroids), also activate downstream signalling cascades that modify protein expression (Zulian *et al.* 2013). These chronic ouabain-induced effects are blocked by digoxin (Zulian *et al.* 2013), which is a ouabain antagonist (Huang *et al.* 1999; Manunta *et al.* 2000; Song *et al.* 2014). This suggests that novel digoxin analogues might block the actions of ouabain without inhibiting Na^+ transport; such agents might be therapeutically useful. Indeed, one such agent, rostafuroxin, was synthesized from digoxigenin (Quadri *et al.* 1997). It blocks the actions of ouabain at concentrations that do not inhibit the Na^+,K^+-ATPase (Ferrari *et al.* 1998; Song *et al.* 2014), and lowers BP in ouabain hypertensive rats and Milan hypertensive rats (Ferrari *et al.* 1998, 1999). Unfortunately, rostafuroxin's affinity for the vascular myocyte Na^+ pump may be too low to be clinically useful (Song *et al.* 2014).

Functional linkage of arterial α2 Na^+ pumps and NCX1. The above findings emphasize the functional (and structural) linkage between $\alpha2$ and NCX1 in arterial myocytes. This cross-talk probably occurs via alterations in $[Na^+]_{SPM}$, which may also be influenced by other adjacent channels and transporters such as TRPC6 (Fig 1*D*) (Arnon *et al.* 2000*a*; Poburko *et al.* 2007, 2008).

Genetic engineering studies illustrate a crucial difference between primary alteration of $\alpha2$ expression and of NCX1 (and SERCA2) expression. Reduction of $\alpha2$ by heterozygous null mutation elevates cell Ca^{2+} and induces hypertension and secondary reduction of NCX1 and SERCA2 expression; the latter is, presumably, a compensatory effect (Zhang *et al.* 2005; Chen *et al.* 2015*b*). Conversely, transgenic overexpression of SM-$\alpha2$ lowers BP and causes a secondary increase in NCX1 and

SERCA2 expression, probably to compensate, partially, for the BP reduction which may be due to a fall in $[Ca^{2+}]_{CYT}$ (Pritchard *et al.* 2007; Chen *et al.* 2015*b*). In contrast, primary SM-NCX1 overexpression increases $[Ca^{2+}]_{CYT}$ and elevates BP (Iwamoto *et al.* 2004), whilst SM-specific knockout of NCX1 lowers $[Ca^{2+}]_{CYT}$ and BP (Zhang *et al.* 2010*b*; Wang *et al.* 2015). We infer that the EO-induced, α2-mediated increase in arterial NCX1 and SERCA2 expression, observed in many types of hypertension (Blaustein & Hamlyn, 2010; Blaustein *et al.* 2012; Pulina *et al.* 2013), contributes directly to the elevation of BP (Chen *et al.* 2015*b*).

How the brain talks to the arteries. In many forms of hypertension, including salt-sensitive hypertension, a central angiotensinergic pathway ('brain RAAS') is activated (Allen, 2011; Gabor & Leenen, 2012; Takahashi, 2012). Circulating Ang II, which is elevated in some forms of hypertension, also stimulates the brain RAAS via circumventricular organs such as the subfornical organ (SFO) (Huang *et al.* 2010; Biancardi *et al.* 2014; Ufnal & Skrzypecki, 2014). This increases CNS driven arterial sympathetic nerve activity (SNA) and α-adrenergic arterial constriction (Fink & Bruner, 1985; Osborn *et al.* 2007, 2011; Gabor & Leenen, 2012; Leenen, 2014), and contributes to BP elevation (Wang *et al.* 2013). Persistent activation of this central angiotensinergic mechanism appears to depend upon a novel neurohumoral pathway that is triggered by high dietary salt/Na^+-rich CSF (Huang *et al.* 2006), as well as Ang II (Huang *et al.* 2010). The hypothalamic component of the neurohumoral pathway involves local aldosterone production, mineralocorticoid receptors, ENaCs, local EO release and α2 Na^+ pumps (Huang & Leenen, 1999; Van Huysse & Hou, 2004; Leenen, 2010; Gabor & Leenen, 2012; Van Huysse *et al.* 2012; Takahashi, 2012). This 'brain EO' enhances hypothalamic Ang type 1 receptor (AT_1R) signalling (Huang *et al.* 2011).

Sustained neurohumoral pathway activation raises circulating EO, which increases arterial expression of NCX1 and SERCA2 (Hamlyn *et al.* 2014); this should enhance arterial responses to sympathetic drive. Elevation of plasma EO and up-regulation of arterial Ca^{2+} transporters, as well as the elevation of BP, are prevented by directly blocking the central neurohumoral pathway (Hamlyn *et al.* 2014). This implies that the increased SNA and the neurohumoral pathway that enhances arterial Ca^{2+} signalling operate jointly to raise BP chronically when the brain angiotensinergic mechanisms are activated.

α2 Na^+ pumps and cardiac function

α2 Na^+ pumps mediate the cardiotonic response to CTS. The positive inotropic effect of CTS on the heart (Fig. 4*A*, *B* and *E*), analogous to the previously mentioned vasotonic effect, requires both ouabain-sensitive Na^+ pumps (see Table 1) and NCX1 (Reuter *et al.* 2002; Dostanic *et al.* 2003, 2004; Altamirano *et al.* 2006). Inhibition of cardiac Na^+ pumps by CTS, the presumed consequent rise in $[Na^+]_{SPM}$ (see Swift *et al.* 2010), and the Na^+-dependent, NCX-mediated net gain of intracellular Ca^{2+} and enhanced Ca^{2+} signalling (Swift *et al.* 2007, 2010) are widely accepted as the basis of the cardiotonic response. Both α1 and α2 Na^+ pumps are located in rodent cardiac muscle t-tubules at or near PM–SR junctions (Mohler *et al.* 2003; Dostanic *et al.* 2004; Berry *et al.* 2007), but which isoform mediates this cardiotonic response? To obtain a definitive answer, engineered 'SWAP' mice (with ouabain-sensitive α1 and resistant α2 pumps, $\alpha1^{S/S}$-$\alpha2^{R/R}$) and WT mice (with ouabain-resistant α1 and sensitive α2 pumps, $\alpha1^{R/R}$-$\alpha2^{S/S}$) were compared. SWAP mice exhibited a positive inotropic response to ouabain that was mediated by the mutated, ouabain-sensitive α1 pumps (Dostanic *et al.* 2004). Nevertheless, comparable inhibition (~25%) of total Na^+ pump activity by low dose ouabain in WT and SWAP mice demonstrates that the α2 isoform preferentially modulates SR Ca^{2+} release and Ca^{2+} transients in cardiomyocytes (Fig. 4*A* and *B*) (Despa *et al.* 2012). Swift and colleagues came to the same conclusion by showing that cardiac α2 pumps and NCX1 are functionally coupled via $[Na^+]_{SPM}$ (Swift *et al.* 2007, 2010). Importantly, despite this compelling evidence, low dose ouabain-induced elevation of $[Na^+]_{SPM}$ (Fig. 4*B*) has not yet been measured (Swift *et al.* 2010). Furthermore, these observations imply that Na^+ diffusion between the sub-PM microdomains and bulk cytosol is restricted (Wendt-Gallitelli *et al.* 1993; Arnon *et al.* 2000*b*; Silverman *et al.* 2003; Poburko *et al.* 2007; Swift *et al.* 2010; Aronsen *et al.* 2013), but definitive data are still lacking.

Cardiac hypertrophy and failure induced by pressure overload: role of α2 Na^+ pumps. Mouse models with genetically engineered α2 Na^+ pumps (Table 1) or altered pump regulation elucidate the link between Na^+ pump expression/activity and cardiac function, and provide new clues to the pathogenesis of heart hypertrophy (HH) and HF. Pressure overload induced by trans-aortic constriction (TAC) is a common model for inducing HH and HF. TAC induces progressive HH and left ventricular (LV) dysfunction in WT mice that depends on the extent and duration of the TAC (Liao *et al.* 2002). Cardio-specific knockout of α2 delays the development of TAC-induced cardiac dysfunction, i.e. increased end-diastolic and systolic volumes, and decreased ejection fraction (EF) (Rindler *et al.* 2013). However, cardio-specific α2, but not α1, overexpression also attenuates TAC-induced HH (Correll *et al.* 2014). How can we reconcile these contradictory results?

First, consider the effects of TAC in mice with altered Na^+ pump ouabain sensitivity. SWAP ($\alpha1^{S/S}$-$\alpha2^{R/R}$) mice

are more susceptible to HH following TAC than are WT ($\alpha1^{R/R}$-$\alpha2^{S/S}$) or $\alpha1^{R/R}$-$\alpha2^{R/R}$ mice, even though the latter two lines have higher LV systolic pressures (Wansapura *et al.* 2011). Heart weight was greatly increased in SWAP mice, but only modestly in WT and $\alpha1^{R/R}$-$\alpha2^{R/R}$ mice, after 4 weeks of TAC. SWAP mice also had substantial LV enlargement, and a reduced EF, indicating cardiac decompensation (HF), i.e. the pathophysiological processes were accelerated. Remarkably, banded $\alpha1^{R/R}$-$\alpha2^{R/R}$ mice had no LV enlargement and no echocardiographic evidence of cardiac dysfunction (*vs.* sham) after 4 weeks of TAC (Wansapura *et al.* 2011). Clearly, TAC-induced HH and HF depend, in part, upon ouabain sensitivity. Further, the cardiac changes are attenuated by anti-ouabain fab fragments (Wansapura *et al.* 2011). Thus, Na^+ pumps *and* their endogenous ligand contribute to the pathogenesis of HH and HF. More rapid TAC-induced cardiac dysfunction is therefore anticipated in SWAP mice because low CTS concentrations inhibit only cardiac $\alpha2$ pumps in WT mice, and ouabain-sensitive $\alpha1$ Na^+ pumps in SWAP mice, and the $\alpha1$:$\alpha2$ ratio is ∼4:1 in both strains (James *et al.* 1999; Berry *et al.* 2007; Despa & Bers, 2007). Thus, at submaximal EO, more pumps will be inhibited in SWAP than in WT mice. In other words, the TAC-induced cardiac dysfunction correlates with the proportion of Na^+ pumps that is EO sensitive. These considerations also explain why both cardiac-$\alpha2$ knockout (Rindler *et al.* 2013) and overexpression (Correll *et al.* 2014) delay/attenuate TAC-induced cardiac dysfunction. Neither $\alpha2$ nor its ouabain receptor is expressed in knockouts. In over-expressors, more 'reserve' $\alpha2$ pumps/EO receptors are available to keep $[Na^+]_{SPM}$ low when a fraction is blocked by EO.

The pressure overload data suggest that EO, via its cardiotonic effect, contributes to HH with preserved, or even enhanced, cardiac performance, e.g. increased EF (Wansapura *et al.* 2011). Human and rodent HF data infer, however, that the impaired contractility and reduced EF also are linked to high plasma EO (Gottlieb *et al.* 1992; Pitzalis *et al.* 2006; Stella *et al.* 2008; Blaustein *et al.* 2015). How can this be reconciled?

The fact that prolonged ouabain treatment activates protein kinase cascades that modulate cardiac protein expression (Tian & Xie, 2008; Li & Xie, 2009) suggests an explanation. In cultured cardiomyocytes, 30–100 μM ouabain (24–48 h) increases NCX1 expression (Vemuri *et al.* 1989; Müller-Ehmsen *et al.* 2003); indeed, 50 nM ouabain (72 h) is sufficient, but 100 nM digoxin is ineffective (Blaustein *et al.* 2015). Increased cardiac NCX1 and decreased SERCA2 expression, which are common findings in human HF and animal models (Studer *et al.* 1994; O'Rourke *et al.* 1999), contribute to the reduced SR Ca^{2+} stores and attenuated Ca^{2+} signals (Bers & Despa, 2006; Lehnart *et al.* 2009). Therefore, while its acute effect is cardiotonic, chronically elevated ouabain/EO and the enhanced NCX1 and reduced SERCA2 expression should accelerate Ca^{2+} extrusion, and promote $[Ca^{2+}]_{SR}$ decline and progression to hypocontractility and HF (Fig. 4*B* and *C*) (Rodriguez *et al.* 2014). Indeed, partial NCX inhibition restores Ca^{2+} signalling in myocytes from failing hearts (Hobai *et al.* 2004). Importantly, these conclusions need to be tested in other HH and HF models, e.g. coronary artery ligation/myocardial infarction (MI), in $\alpha2^{R/R}$ and SWAP mice.

Regulation of $\alpha2$ pumps in HH and HF. In some forms of HF, expression of $\alpha1$, $\alpha2$ and PLM, and Na^+ pump activity, are all reduced, and $[Na^+]_{CYT}$ is elevated, in left ventricular myocytes (Bossuyt *et al.* 2005; Pavlovic *et al.* 2013*a*), although PLM transcription is up-regulated (Gronich *et al.* 2010). Also, in HF, oxidative stress (Burgoyne *et al.* 2012) inhibits cardiomyocyte Na^+ pumps by inducing $\beta1$ subunit glutathionylation; this can be reversed by phosphorylated PLM (Bibert *et al.* 2011).

The link between $\alpha2$ activity and cardiac pathophysiology is affirmed by two models of PLM dysregulation. In PLM knockout mice, total Na^+,K^+-ATPase activity and $\alpha2$ Na^+ pump expression are reduced by 50–60% (Jia *et al.* 2005). This should sustain a high $[Na^+]_{SPM}$ and a large cardiotonic effect (Golovina *et al.* 2003) to account for the hypertrophic hearts and increased LV EF (Jia *et al.* 2005).

In the second model, mice with non-phosphorylatable PLM (PLM^{35A}) have normal cardiac function under basal conditions. Following TAC, however, PLM^{35A} mice exhibit accelerated cardiac hypertrophy and dysfunction with increased NCX and decreased SERCA2a expression (Boguslavskyi *et al.* 2014). The inability to phosphorylate PLM and augment pump-mediated Na^+ extrusion and, thus, NCX-mediated Ca^{2+} extrusion, when the heart is stressed (e.g. by TAC) enhances Ca^{2+} dysregulation and accelerates the cardiomyopathy. These models reinforce the view that cardiac $\alpha2$ Na^+ pumps and NCX1 conjointly contribute to the pathogenesis of HH and HF. An important caveat, however, is that in the rat heart in HF, expression of $\alpha2$ declines and $\alpha3$, the fetal isoform, increases (Semb *et al.* 1998; Verdonck *et al.* 2003), but the significance of this isoform switch and the localization of $\alpha3$ are unknown.

Myocardial $[Na^+]_{CYT}$ and NCX1 in HF. Elevated myocardial $[Na^+]_{CYT}$ (Pogwizd *et al.* 2003; Murphy & Eisner, 2009; Bay *et al.* 2013; Pavlovic *et al.* 2013*a*) fosters the NCX-mediated Ca^{2+} dysregulation in HF (Bers & Despa, 2006; Despa & Bers, 2013; Shattock *et al.* 2015). Multiple mechanisms may contribute to the high $[Na^+]_{CYT}$, including: (i) reduced $\alpha2$ pump expression; (ii) Na^+ pump dysregulation due to reduced PLM expression (Bossuyt *et al.* 2005); (iii) increased

late Na^+ current due to altered CaMKII regulation of cardiac Na^+ channels (Grandi & Herren, 2014); (iv) direct inhibition of α2 by the elevated plasma EO (Gottlieb *et al.* 1992; Pitzalis *et al.* 2006; Stella *et al.* 2008; Hamlyn & Manunta, 2015); (v) increased Na^+ entry via Na^+/H^+ exchange (Baartscheer *et al.* 2003; Karmazyn *et al.* 2008); (vi) dysregulation of the Ca^{2+}-dependent, nitric oxide (NO)-mediated mechanism that stimulates Na^+ pumps by phosphorylating PLM (Pavlovic *et al.* 2013*b*); and (vii) increased oxidative stress and ROS generation (Munzel *et al.* 2015; Zuo *et al.* 2015) that not only reduces NO availability and PLM phosphorylation, but also increases β1 subunit glutathionylation (Figtree *et al.* 2009); both mechanisms depress pump-mediated cation transport.

Elevated $[Na^+]_{CYT}$ promotes Ca^{2+} export by the mitochondrial Na^+/Ca^{2+} exchanger, NCLX, which lowers intra-mitochondrial $[Ca^{2+}]$ and increases oxidation of mitochondrial NADH (Murphy & Eisner, 2009; Liu *et al.* 2010; De Marchi *et al.* 2014; Nita *et al.* 2015). Thus, elevated $[Na^+]_{CYT}$ and/or $[Ca^{2+}]_{CYT}$ (which should limit NCLX-mediated Ca^{2+} export) can not only enhance Ca^{2+} signalling, but also increase oxidative stress and ROS production (Li *et al.* 2014) and further depress Na^+ pump function (Figtree *et al.* 2009).

The preceding two paragraphs focus on 'global' $[Na^+]_{CYT}$. However, $[Na^+]_{SPM}$, which apparently modulates cardiac $[Ca^{2+}]_{CYT}$ transients and excitation–contraction coupling, may be independently affected (Su *et al.* 2001; Verdonck *et al.* 2004; Swift *et al.* 2010; Aronsen *et al.* 2013). Further, $[Na^+]_{SPM}$ may also be modified by Na^+ channels associated with these microdomains (Verdonck *et al.* 2004; Aronsen *et al.* 2013).

Genetically induced cardiac NCX1 overexpression in mice, itself, accelerates HH and HF induced by stresses (TAC, intense exercise, or pregnancy) known to activate the RAAS (Roos *et al.* 2007). The NCX1 over-abundance and enhanced Ca^{2+} removal are manifested by reduced SR Ca^{2+}, Ca^{2+} transients, and excitation–contraction coupling gain (Reuter *et al.* 2004; Ottolia *et al.* 2013), also observed in myocytes from failing human and rat hearts (Gomez *et al.* 1997; Piacentino *et al.* 2003). In contrast, genetically reduced (50%) cardiac NCX1 expression confers tolerance to pressure overload and attenuates HF development, perhaps by reducing Ca^{2+} overload (Takimoto *et al.* 2002; Jordan *et al.* 2010). Nevertheless, ~20% of WT NCX1 is essential for cardiac function: nearly complete knockout causes HH and accelerates stress-induced progression to HF (Jordan *et al.* 2010), presumably because of Ca^{2+} overload due to impaired Ca^{2+} clearance. NCX1 apparently also plays a role in some other HH and HF models. For example, cardiac-specific NCX1 knockout (by 80–90%) mitigates chronic intermittent hypoxia-induced LV hypertrophy and contractile dysfunction in mice (Chen *et al.* 2010).

Cardiomyocyte Ca^{2+} dysregulation in HF. In HF, cardiac Ca^{2+} dysregulation is usually manifested by elevated diastolic $[Ca^{2+}]_{CYT}$ but reduced SR Ca^{2+} content (Fig. 4*C–E*) (Lehnart *et al.* 2009; Reuter & Schwinger, 2012). The latter, which may be the result of reduced SERCA2 expression and activity (Lehnart *et al.* 2009) and increased Ca^{2+} leakage through ryanodine receptors (RyRs) (Marx & Marks, 2013), probably explains the attenuated peak systolic $[Ca^{2+}]_{CYT}$ transients and cardiac hypocontractility (Fig. 4*D* and *E*). The elevated diastolic (quasi-steady state) $[Ca^{2+}]_{CYT}$ can be attributed largely to the previously mentioned high $[Na^+]_{CYT}$ and reduced driving force for Ca^{2+} extrusion via NCX, although reduced SR Ca^{2+} uptake and increased RyR leak may also contribute. The high $[Na^+]_{CYT}$ and thus $[Ca^{2+}]_{CYT}$ may help explain the impaired relaxation and increased stiffness of cardiac muscle in HF (Kass *et al.* 2004; Louch *et al.* 2010; Li *et al.* 2012).

Central and peripheral mechanisms in the progression from hypertrophy to failure. In HH and HF, as in hypertension, central angiotensinergic mechanisms are usually stimulated, and sympathetic drive is increased (Yu *et al.* 2008; Westcott *et al.* 2009; Lymperopoulos *et al.* 2013; Zucker *et al.* 2014). Blockers of these mechanisms are therefore used to treat both hypertension and HF (Leenen *et al.* 2012; Krum & Driscoll, 2013; James *et al.* 2014). The angiotensinergic mechanisms activate the neurohumoral pathway that elevates circulating EO (Hamlyn *et al.* 2014): e.g. both MI and S.C. Ang II + high dietary salt raise plasma EO and increase both cardiac and arterial NCX1 expression (Blaustein *et al.* 2015). Because arterial NCX1 operates primarily in the Ca^{2+} entry mode, both acute and chronic high EO should enhance vasoconstriction and foster hypertension, but why do high EO and increased NCX1 also lead to cardiac hypocontractility and failure? The main cardiac Ca^{2+} extrusion mechanism, NCX1, exports Ca^{2+} during most of the cardiac cycle because, during diastole, the membrane potential is about −65 to −75 mV (Eisner *et al.* 2013; Eisner, 2014). Acutely elevated plasma EO therefore induces 'classic' positive inotropy, but markedly increased cardiac NCX1 expression, due to chronically elevated EO, promotes Ca^{2+} extrusion, reduces $[Ca^{2+}]_{CYT}$ and causes negative inotropy (Fig. 4*B–D*).

Comparison of WT, $\alpha2^{R/R}$ and SWAP mouse data (Wansapura *et al.* 2011) lead us to postulate that TAC activates the brain RAAS–neurohumoral pathway, raises plasma EO, and in WT, and even more so in SWAP mice ($\alpha2^{R/R}$ mice are EO-resistant), induces a positive inotropic response. This is initially amplified as NCX1 expression increases. The consequent, sustained hypercontractility, as well as other, possibly EO-triggered, changes in protein programming contribute to hypertrophy and, at least initially, to enhanced cardiac performance. With progressive increase in NCX1 and decrease in SERCA2

expression, however, the NCX1-mediated Ca^{2+} extrusion mode starts to prevail, and $[Ca^{2+}]_{CYT}$ falls, thereby reducing cardiac performance and leading to HF; i.e. the cardiac changes in HH and HF are a continuum. Indeed, HF with preserved EF (Kamimura *et al.* 2012; Gladden *et al.* 2014; Sharma & Kass, 2014; Zuo *et al.* 2015) might be an intermediate stage in this continuum. Further, following MI, even the RAAS-stimulated initial tendency to induce a positive inotropic response may be circumvented rapidly if there is much damaged and unresponsive myocardium. The altered NCX1 and SERCA2 expression may then dominate early on, leading rapidly to a negative ionotropic response and HF (Fig. 4*D*).

Exercise intolerance in HF: role of skeletal muscle α2 Na^+ pumps in fatigue resistance. Skeletal muscle (SkM) α2 plays a negligible role in quiescent muscle, but is activated by the rise in t-tubule $[K^+]$ and $[Na^+]_{CYT}$ during exercise, and is needed to attenuate fatigue (DiFranco *et al.* 2015; Manoharan *et al.* 2015). As in the heart, phosphorylation of SkM PLM enhances Na^+ pump activity, but PLM knockout mice show that PLM is not needed for acute exercise-induced SkM α2 activation (Manoharan *et al.* 2015). Nevertheless, intense exercise increases PLM phosphorylation, and α2 pump expression and activity in human type II (fast twitch, fatigable) SkM fibres, which express more α2 than do type I (slow twitch, fatigue-resistant) fibres (Kristensen *et al.* 2008; Thomassen *et al.* 2010, 2013; Benziane *et al.* 2011). Reduced α2 Na^+ pump expression in ageing humans may decrease muscle strength and increase fatigability (Chibalin *et al.* 2012).

Mice with targeted knockout of SkM α2 pumps, $\alpha2^{SkM-/-}$, fatigue faster than WT mice on a treadmill (Radzyukevich *et al.* 2013). Also, extensor digitorum longus (EDL) muscles isolated from SkM-$\alpha2^{-/-}$ mice have reduced twitch and tetanic force compared to WT EDL. Selective block of α2 by ouabain in WT EDL mimics the results in $\alpha2^{SkM-/-}$ EDL (Radzyukevich *et al.* 2013). Resistance to fatigue apparently is due in part to the rapid increase in α2-mediated cation transport triggered by the rise in t-tubule $[K^+]$ during stimulation (DiFranco *et al.* 2015).

α2 ouabain binding sites and EO play a role in SkM: $\alpha2^{R/R}$ mice exhibit fewer exercise failures on a treadmill than do WT mice (Radzyukevich *et al.* 2009). Also, ^{86}Rb (K^+ surrogate) uptake is reduced following high frequency contractile activation (*vs.* rest) in EDL from WT mice. In contrast, following muscle stimulation, ^{86}Rb uptake is increased in EDL from $\alpha2^{R/R}$ mice and in EDL from WT mice pre-infused with DigiBind prior to euthanasia (Radzyukevich *et al.* 2009).

Clearly, susceptibility to fatigue is inversely related to skeletal muscle α2 Na^+ pump expression/activity and is modulated by EO, but why? A clue is that knockout of the predominant NCX isoform in SkM, NCX3, also reduces endurance and increases fatigue, although it increases both twitch and tetanus tension (Sokolow *et al.* 2004). We postulate that reduced NCX3-mediated Ca^{2+} extrusion, due to decreased NCX3 expression or diminished Na^+ extrusion by α2 pumps when exercise elevates t-tubule $[K^+]$, enhances fatigability.

The above findings imply that the high EO levels observed in hypertension and HF contribute to the reduced SkM α2 Na^+ pump activity (contrast Barr *et al.* 2005), increased fatigability (Carlsen *et al.* 1996; Helwig *et al.* 2003; Okita *et al.* 2013; Tzanis *et al.* 2014) and reduced hand grip strength (Mainous *et al.* 2015). Exercise may enhance SkM α2 Na^+ pump activity by increasing α2 expression or translocation to the sarcolemma, or PLM phosphorylation, and thereby reduce fatigability and improve muscle strength (Thomassen *et al.* 2010; Rasmussen *et al.* 2011).

Summary and conclusions

Mice with genetically engineered α2 Na^+ pumps, PLM and NCX1 provide novel insight into the central role of α2 and its endogenous ligand, EO, in regulating Ca^{2+} homeostasis and the function of cardiac, skeletal and vascular muscles. The juxtaposition of these findings enables us to recognize the striking similarities and key differences between the mechanisms involved in the pathogenesis of hypertension, HH and HF. In all three situations, brain angiotensinergic mechanisms are activated; this triggers the CNS rapid sympathetic and slower neurohumoral (EO-mediated) pathways. Acutely increased nerve frequency is often attenuated by self-tuning (Greengard, 2001; Turrigiano, 2008), but EO may potentiate peripheral synaptic transmission and sympathetic nerve responses (Aileru *et al.* 2001). Also, the chronic, protein kinase cascade-mediated effects of elevated plasma EO on arteries and heart may amplify the cardiac and vascular responses to sympathetic drive. Initially, the cardio- and vasotonic actions of EO enhance Ca^{2+} signalling and contractility and thus elevate BP and heighten cardiac function, and lead to hypertrophy. Slow, EO-mediated up-regulation of NCX1 (and SERCA2) in arteries favours Ca^{2+} entry and further fosters vasoconstriction. In the heart, however, EO-mediated NCX1 up-regulation (and SERCA2 decline) eventually tips the balance toward Ca^{2+} exit, hypocontractility and HF. It is worth emphasizing that, when the brain RAAS is activated in hypertension, the elevated plasma EO is expected to influence cardiac function simultaneously. Likewise, when an MI activates the brain RAAS, simultaneously altered arterial function is expected (Blaustein *et al.* 2015). Thus, elevated plasma EO is likely to contribute to the increased peripheral vascular resistance often observed in HF post-MI (Zelis *et al.* 1968; Ledoux *et al.* 2003).

Despite this compelling evidence for the key roles of α2 and EO, numerous challenges remain. First of all, details of the CNS pathways are poorly understood. For example, brain α2 pumps are important in SS-hypertension (Leenen *et al.* 2015), but the cellular location of the relevant pumps is unknown. Also, the proposed role of brain α2 in HH and HF must be verified. Further, while EO is synthesized in the brain, and is a critical link in both hypertension and HF (Leenen *et al.* 1995; Huang *et al.* 2010), precisely where in the CNS pathways it participates is unresolved.

Circulating EO comes from the adrenals (Hamlyn *et al.* 1991; Boulanger *et al.* 1993; Manunta *et al.* 2010), but what is the biosynthetic pathway? Also, how does brain RAAS regulate plasma EO? Is it via increased sympathetic traffic to the adrenals (Shah *et al.* 1998), or some other mechanism? And, what role, if any, does ACTH play (Laredo *et al.* 1994)?

We suggest that α2 pumps mediate both the acute and chronic effects of nanomolar ouabain/EO in rodents (Dostanic *et al.* 2005; Despa *et al.* 2012; Zulian *et al.* 2013), but others suggest that α1 pumps are responsible for the chronic effects (Liu & Xie, 2010; Xie *et al.* 2015). Comparison of the acute and chronic effects of nanomolar ouabain on WT and $\alpha2^{R/R}$ cardiac and arterial myocytes could resolve this controversy. Further, since human α1 is ouabain sensitive, do human α2 pumps play the same key role as in rodents? A clue is that human, like rodent, arterial α2 is localized in PM microdomains at PM–S/ER junctions (Linde *et al.* 2012).

We postulate that the $[Na^+]_{SPM}$ at PM–S/ER junctions is a crucial factor in the EO-dependent modulation of Ca^{2+} signalling and contractility in the arteries and heart (Figs 3 and 4). More precise information about the structural organization of the junctions and their resident transporters, e.g. using super-resolution imaging, should improve our understanding of ion regulation in these regions. Critically, direct measurement of the effects of nanomolar ouabain on $[Na^+]_{SPM}$ with Na^+-sensitive fluorochromes and, e.g., 'total internal reflection fluorescence' (TIRF) imaging, is needed to validate our inferences.

The effect of sustained ouabain/EO exposure on signalling cascades is established, but the precise time course of these responses, and all of the contributors (e.g. the complete range of affected Ca^{2+} transporter and signalling molecules), are unknown. For example, does ouabain/EO, *per se*, trigger down-regulation of cardiac SERCA2 expression? Measurement of ouabain/EO- and disease-dependent gene activation (quantitative PCR analysis of mRNA) or changes in protein expression (MS) would provide important new clues to underlying pathogenic mechanisms.

Finally, a fundamental implication of the work reviewed above is that novel agents that interfere with the biosynthesis, release and/or peripheral actions of EO should be therapeutically beneficial in hypertension, HH and HF. Such agents might also be useful in attenuating the renal damage, often linked to these CV diseases, that has been attributed to elevated circulating EO (Bignami *et al.* 2013; Ferrandi *et al.* 2014; Hamlyn & Manunta, 2015). Indeed, application of these agents would provide a critical test of many of the ideas summarized here.

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Additional information

Competing interests

None declared.

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