Title

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Journal

The Journal of Physiology, 593(6)

ISSN

0022-3751

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Publication Date

2015-03-15

DOI

10.1113/jphysiol.2014.282319

Peer reviewed

J Physiol 593.6 (2015) pp 1361–1382

The Journal of Physiology

WHITE PAPER

Na^+/Ca^{2+} exchange and Na^+/K^+-ATPase in the heart

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Abstract This paper is the third in a series of reviews published in this issue resulting from the University of California Davis Cardiovascular Symposium 2014: *Systems approach to understanding cardiac excitation–contraction coupling and arrhythmias: Na^+ channel and Na^+ transport*. The goal of the symposium was to bring together experts in the field to discuss points of consensus and controversy on the topic of sodium in the heart. The present review focuses on cardiac Na^+/Ca^{2+} exchange (NCX) and Na^+/K^+-ATPase (NKA). While the relevance of Ca^{2+} homeostasis in cardiac function has been extensively investigated, the role of Na^+ regulation in shaping heart function is often overlooked. Small changes in the cytoplasmic Na^+ content have multiple effects on the heart by influencing intracellular Ca^{2+} and pH levels thereby modulating heart contractility. Therefore it is essential for heart cells to maintain Na^+ homeostasis. Among the proteins that accomplish this task are the Na^+/Ca^{2+} exchanger (NCX) and the Na^+/K^+ pump (NKA). By transporting three Na^+ ions into the cytoplasm in exchange for one Ca^{2+} moved out, NCX is one of the main Na^+ influx mechanisms in cardiomyocytes. Acting in the opposite direction, NKA moves Na^+ ions from the cytoplasm to the extracellular space against their gradient by utilizing the energy released from ATP hydrolysis. A fine balance between these two processes controls the net amount of intracellular Na^+ and aberrations in either of these two systems can have a large impact on cardiac contractility. Due to the relevant role of these two proteins in Na^+ homeostasis, the emphasis of this review is on recent developments regarding the cardiac Na^+/Ca^{2+} exchanger (NCX1) and Na^+/K^+ pump and the controversies that still persist in the field.

(Received 8 August 2014; accepted after revision 30 October 2014)

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Abbreviations CKD, chronic kidney disease; CTS, cardiotonic steroids; EC, excitation–contraction; EGFR, epidermal growth factor receptor; ESRD, end-stage renal disease; FRET, fluorescence resonance energy transfer; NCX, Na^+/Ca^{2+} exchanger; NKA, Na^+/K^+-ATPase (Na^+/K^+ pump); PKA, protein kinase A; PKC, protein kinase C; PLM, phospholemman; RYR, ryanodine receptor; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TMS, transmembrane segment; VSM, vascular smooth muscle.

M. J. Shattock and M. Ottolia contributed equally to this work.

DOI: 10.1113/jphysiol.2014.282319

The Na^+/Ca^{2+} exchanger – structure, function and regulation

The session on 'Na/Ca exchanger – structure, function and regulation' included Michela Ottolia, Karin Sipido, John Bridge, Joshua Goldhaber, Andrew Edwards and Donald Bers as speakers, discussion leaders and panelists. Below is a summary of the current state of knowledge on the Na^+/Ca^{2+} exchanger in cardiac ion homeostasis.

Molecular perspectives. The eukaryotic exchanger protein, as exemplified by the mammalian cardiac isoform NCX1.1 (indicated as NCX throughout the review), is organized into ten transmembrane segments (TMSs) (Liao *et al.* 2012; Ren & Philipson, 2013) with a large cytoplasmic loop between TMSs 5 and 6 that plays a regulatory role (Philipson *et al.* 2002). Ion transport is associated with two regions of intramolecular similarity named α repeats (Nicoll *et al.* 1996; Schwarz & Benzer, 1997; Shigekawa *et al.* 2002; Ottolia *et al.* 2005). They consist of TMSs 2–3 and TMSs 7–8 and their connecting links. The α repeats are highly conserved within the exchanger family to the extent that they are deemed the signature sequence of the Ca^{2+}/cation exchanger class of proteins (Lytton, 2007). Their role in ion translocation has been confirmed by mutagenesis studies (Nicoll *et al.* 1996; Schwarz & Benzer, 1997; Shigekawa *et al.* 2002; Ottolia *et al.* 2005) and recently by the structure of the archaebacterial homologue NCX_Mj (Liao *et al.* 2012) (Fig. 1). In NCX_Mj the two α repeats are packaged in the core of the protein and form four ion binding sites (three for Na^+ and one of Ca^{2+}) which are reachable via two translocation pathways (Fig. 1). Essential amino acids responsible for Na^+ and Ca^{2+} ion binding are conserved between the archaea and eukaryotic proteins and accessibility data indicate that the portions of α repeats are organized in a similar manner in the mammalian cardiac exchanger (John *et al.* 2013). These similarities are indicative of functional and mechanistic conservation between NCX_Mj and NCX. However, enthusiasm is tempered by the low sequence similarity between these two proteins outside the α repeats and the usefulness of NCX_Mj as a structural guide to investigate the properties of the mammalian homologue should be further investigated. For example, the links between TMSs 2 and 3 and TMSs 7 and 8 of the α-repeats appear to fold differently in the archaebacterial and mammalian exchangers (Fig. 1). Previous accessibility mapping studies using cysteine scanning mutagenesis and epitope tagging (Nicoll *et al.* 1996; Iwamoto *et al.* 1999, 2000; Shigekawa *et al.* 2002) indicate that these two portions of the cardiac NCX form re-entrant loops while the homologous regions in NCX_Mj (Liao *et al.* 2012) lack the re-entrant loop between TMSs 2 and 3 and have an additional transmembrane segment between TMSs 7 and 8 (Fig. 1). Many of these residues are intimately involved in ion transport as determined by mutagenesis studies.

Regulation of the mammalian NCX has been elegantly demonstrated both at the functional and structural level. Allosteric regulation of NCX by cytoplasmic Na^+ and Ca^{2+} ions occurs from within the large cytoplasmic loop that separates TMS 5 from TMS 6 (Philipson *et al.* 2002). This regulatory loop is absent from the NCX_Mj structure. However, the structures of two regulatory domains within this region of the eukaryotic exchanger are now available (Hilge *et al.* 2006; Nicoll *et al.* 2006; Besserer *et al.* 2007; Wu *et al.* 2009*a*,*b*, 2013). These two contiguous stretches of residues bind cytoplasmic Ca^{2+} and are identified as Ca^{2+} binding domains 1 and 2, respectively. They both adopt a β-sandwich fold organized in a head to tail fashion separated by only 5–7 amino acids. Binding of Ca^{2+} to these domains increases NCX activity (Hilgemann *et al.* 1992; Matsuoka *et al.* 1995; Ottolia *et al.* 2009) via molecular mechanisms not yet fully understood. The role of Ca^{2+} regulation *in vivo* is also uncertain as it is difficult to separate the effects of

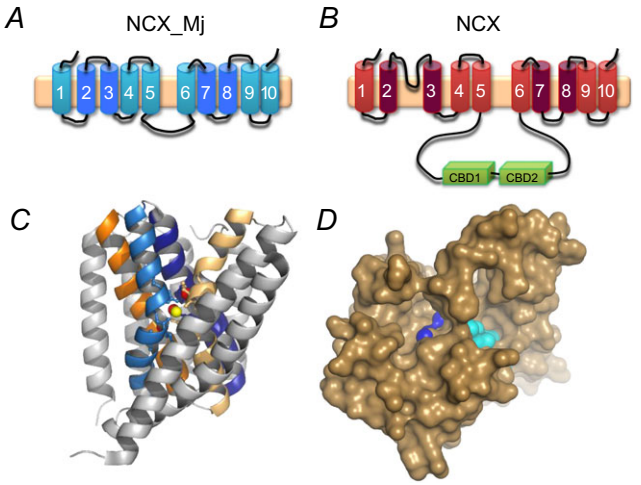

Figure 1. Molecular properties of the Na^+/Ca^{2+} exchanger
The upper panels show the secondary topologies of the archaebacterial (*A*) and eukaryotic (*B*) exchangers. The conserved α-repeats (TMSs 2–3 and 7–8) are highlighted in darker shades. Note that the loop connecting TMS2 and TMS3 is modelled as a P loop in the eukaryotic NCX (*B*). The atomic structure of the archaebacterial NCX (NCX_Mj) (Liao *et al.* 2012) is shown in *C*. The transmembrane segments involved in ion transport are TMSs 2, 3, 7 and 8, coloured in cyan, dark blue, yellow and orange, respectively. The cytoplasmic ion translocation pathways of the cardiac exchanger have been investigated by cysteine scanning mutagenesis (John *et al.* 2013). A comparison between these data and the structure of NCX_Mj suggests that these two proteins have similar architecture. This is shown in *D* where the residues (shown in cyan and blue) of the cardiac exchanger found sensitive to thiol modification were mapped onto NCX_Mj inward structural model (shown as surface representation as seen from the cytoplasmic side). The accessibility data obtained from the eukaryotic exchanger correlates well with the NCX_Mj structure.

Ca^{2+} on the transport *vs.* regulatory sites. Recent evidence suggests that while Ca^{2+} ions bind rapidly to these sites at physiological levels (10–300 nM), its release is slow suggesting that *in vivo* NCX may be persistently activated during excitation–contraction (EC) coupling (Reeves & Condrescu, 2003; Bers & Ginsburg, 2007; Giladi *et al.* 2010; Ginsburg *et al.* 2013; John *et al.* 2013). Na^+ ion regulation of NCX is less well studied. High cytoplasmic Na^+ inactivates the Na^+/Ca^{2+} exchanger (Hilgemann *et al.* 1992) consistent with a state-dependent model, but the mechanism is elusive. One hypothesis is that the binding of Na^+ to its transport site drives the exchanger into an inactivated state (Hilgemann *et al.* 1992). However, mutagenesis studies have shown that a region within the large cytoplasmic loop is also involved in this process (Matsuoka *et al.* 1995). Whether NCX modulation by cytoplasmic Na^+ is relevant to cardiac physiology remains to be established since relatively high intracellular Na^+ concentrations ($\geq$20 mM) are required to significantly inactivate NCX experimentally (Hilgemann *et al.* 1992; Matsuoka & Hilgemann, 1994; Matsuoka *et al.* 1995). Possibly, elevations in Na^+ levels due to pathological conditions such as heart failure (Despa *et al.* 2002*b*; Despa & Bers, 2013) could influence NCX activity via Na^+-dependent inactivation. Further investigations into NCX regulation by Na^+ and Ca^{2+} are essential to determine the physiological impact of NCX on heart contractility.

Question or controversy: molecular perspectives

The crystal structure of the archaebacterial Na^+/Ca^{2+} exchanger represents a milestone in the exchanger field and revealed, unexpectedly, the absence of re-entrant loops considered important for ion translocation in the eukaryotic homologues. Due to the low sequence similarity between the eukaryotic and archaebacterial exchanger, further studies are required to elucidate the properties of the mammalian NCX and to address the following questions:

Does the mammalian NCX differ structurally from the archaebacterial exchanger?

Evidence indicates that the mammalian NCX exists as a dimer in the membrane (Ren *et al.* 2008; John *et al.* 2011) while the archaebacterial exchanger crystalizes as a monomer. Does the oligomeric state of NCX affect its function?

NCX in excitation–contraction coupling. The Na^+/Ca^{2+} exchanger catalyses the countertransport of three Na^+ and one Ca^{2+} using the energy of the Na^+ gradient as driving force. The cytoskeletal protein ankyrin B anchors NCX to the membrane and the NKA may be part of this complex suggesting the proximity of these two proteins (Mohler *et al.* 2005). In cardiomyocytes NCX appears widely distributed within the sarcolemma but there has been some controversy concerning the proportion of NCX molecules located in T-tubules (Frank *et al.* 1992; Despa *et al.* 2003), which would have considerable implications for EC coupling. For example, confocal and internal reflection imaging (Jayasinghe *et al.* 2009) show that 27% of NCX labelling in rat myocytes is within 150 nm of ryanodine receptor (RYR) puncta and that 45% of all NCX labelling is concentrated within puncta. As the authors explain these could be viewed as sites of concentrated Ca^{2+} entry or exit and is consistent with a fraction of NCX being located within couplons, i.e. junctions. This is consistent with earlier electrophysiological data suggesting that NCX

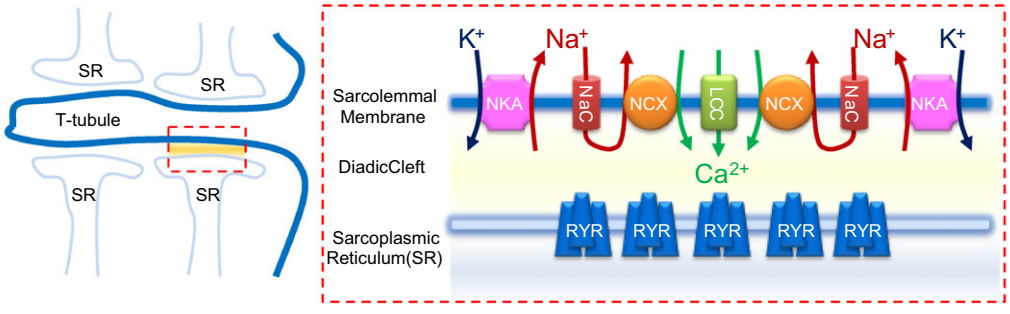

Figure 2. The Na^+/Ca^{2+} exchanger primes the diadic cleft with Ca^{2+}

The left panel shows the organization of the T-tubule and sarcoplasmic reticulum (SR) in an adult cardiac myocyte. The specialized junction between the T-tubule and the SR is highlighted in the red box. This region is further detailed on the right panel which depicts proteins important for excitation–contraction (EC) coupling (NKA, Na^+/K^+ pump; NCX, Na^+/Ca^{2+} exchange; NaC, voltage-dependent Na^+ channels; LCC, voltage-dependent Ca^{2+} channels; RYR, ryanodine receptor). Activation of the voltage-dependent Na^+ channels increases the Na^+ concentration in proximity of the Na^+/Ca^{2+} exchanger, promoting its reverse mode. As a result, Ca^{2+} is transported into the diadic cleft, priming EC coupling by facilitating the opening of RYRs by Ca^{2+} entering through voltage-dependent Ca^{2+} channels.

can sense subsarcolemmal Ca^{2+} during sarcoplasmic reticulum (SR) release (Trafford *et al.* 1995; Weber *et al.* 2002); the dual time course of the NCX current during caffeine-induced SR Ca^{2+} release in rat myocytes is consistent with a fraction of NCX sensing early and high local Ca^{2+} (in the dyad) and another being activated with a delay (outside the dyad). Recent data indicate that L-type Ca^{2+} channels and NCX within the dyadic cleft report similar microdomain Ca^{2+} levels, with approximately 15% of all NCX located within close proximity to RyR in pig ventricular myocytes (Acsai *et al.* 2011). Besides results indicating that the triggering of SR Ca^{2+} release flux requires NCX which is activated by triggering Ca^{2+} current (Sobie *et al.* 2008), there is also evidence indicating that Na^+ entry, via voltage-dependent Na^+ channels, can drive NCX into the reverse mode thereby priming the dyadic cleft with a small amount of Ca^{2+}, thus enhancing triggering by voltage-dependent Ca^{2+} channels (Fig. 2) (Larbig *et al.* 2010; Neco *et al.* 2010; Torres *et al.* 2010). Abrupt inhibition of NCX-mediated Ca^{2+} extrusion can instantaneously increase the frequency of Ca^{2+} sparks in resting cardiac myocytes (Goldhaber *et al.* 1999).

Taken together, several lines of evidence suggest that a fraction of NCX is within the junctional space of the dyadic cleft. More studies to confirm the precise fraction and location of NCX within the junctional region would be of considerable value and will doubtless be forthcoming as the methods of high resolution confocal microscopy are refined. The data obtained in rodent models further need to be complemented with data from human and large mammal models which have a low T-tubular density and a large fraction of RyRs that are not within the dyadic cleft (Louch *et al.* 2004). Importantly, in disease, reorganization of the membrane structure and dyadic cleft occur and affect NCX interaction with RyR (Biesmans *et al.* 2011).

The Na^+/Ca^{2+} exchanger is the major cardiac extrusion mechanism for the Ca^{2+} that enters via voltage-dependent Ca^{2+} channels with each beat. In conjunction with SR Ca^{2+} uptake, Ca^{2+} removal by NCX allows myocytes to relax. Concurrently, the coupled inward depolarizing Na^+ current is a fundamental component of the action potential duration (Armoundas *et al.* 2003). Moreover, outward shifts in both NCX and NKA current at higher $[Na^+]_i$ are important in determining action potential repolarization and duration (Grandi *et al.* 2010). In these roles, NCX is an important determinant of intracellular Ca^{2+} and Na^+ and hence contractility. Direct evidence of the essential role of NCX in EC coupling comes from ventricular-specific conditional knockouts of NCX. Two main adaptive mechanisms appear relevant for the survival of these mice lacking an exchanger to provide Ca^{2+} efflux: (i) a reduction in Ca^{2+} influx via voltage-dependent Ca^{2+} channels, and (ii) an increase in the gain in the EC coupling; both of these are probably due to an increase in Ca^{2+} within the dyadic cleft (Pott *et al.* 2005).

In addition to maintaining both proper contraction and Na^+ homeostasis, NCX has also been implicated in the generation of the pacemaker potential within the sino-atrial node (SA node). Rhythmic spontaneous release of Ca^{2+} from the SR via the RYR activates NCX resulting in a net inward current facilitating depolarization helping the pacemaker cells reach the threshold required for triggering an action potential. Several models of mice lacking NCX within the pacemaker cells have been generated with the goal of determining the role of NCX in the genesis of pacemaker activity (Gao *et al.* 2013; Groenke *et al.* 2013; Herrmann *et al.* 2013). Complete ablation of NCX from both the atria and SA node tissue abolishes atrial depolarization and sinus rhythm in an atrial-specific NCX knockout (KO) mouse (Groenke *et al.* 2013). Isolated SA node cells from these NCX KO mice are viable, but they have no spontaneous action potentials despite an intact funny current (I_f) through HCN4 channels. This clearly demonstrates the importance of NCX in SA node pacemaking.

Question or controversy: NCX in excitation–contraction coupling

The role of NCX in priming EC coupling is well documented but important questions remain:

Does NCX localize within the dyadic cleft? If so, what is the ratio of NCX in the cleft to those outside?

Does NCX interact with other proteins involved in EC coupling such as NKA or Na^+ channels?

How does NCX prime the dyadic cleft to facilitate EC coupling?

NCX and heart pathologies. Under physiological conditions NCX ensures that intracellular Ca^{2+} is removed after each beat to allow for proper relaxation and cellular Ca^{2+} balance. However, common pathologies promote Ca^{2+} influx via NCX leading to Ca^{2+} accumulation which can accelerate myocardial necrosis, contractile failure and also trigger arrhythmias. This is seen for example during reperfusion after an ischaemic event when acidosis provokes Na^+ overload by the Na^+/H^+ exchanger and slows or even reverses NCX. This example clearly demonstrates the tight coupling between Na^+ and Ca^{2+} homeostasis.

Alterations in NCX expression are also associated with various cardiac pathologies including heart failure and post-ischaemic cardiac injury. Heart failure, a highly prevalent condition world-wide, is mostly due to ischaemic heart disease, valvular heart disease and hypertension. It is associated with high mortality as a result of contractile dysfunction and ventricular arrhythmia. While evidence indicates that NCX expression and function are significantly increased during the course of this pathology (Studer *et al.* 1994; Dipla *et al.* 1999; Pogwizd *et al.* 1999;

Hobai & O'Rourke, 2000), this is not a characteristic of all stages of the disease nor in all types of heart failure (reviewed in Sipido *et al.* 2002; Antoons *et al.* 2012). The NCX function ultimately depends on prevailing Ca^{2+} and Na^+ concentrations, as well as NCX distribution in the membrane and surface-to-volume changes. Increased NCX inward current in response to spontaneous SR Ca^{2+} release enhances afterdepolarizations, while the additional Ca^{2+} efflux reduces SR Ca^{2+} load. These events contribute to the arrhythmogenicity and depressed contractility associated with heart failure (Pogwizd *et al.* 1999; Pogwizd & Bers, 2002). Additionally, an increase in intracellular Na^+ has been reported in cardiac myocytes during hypertrophy and heart failure with important implications for NCX activity and thereby on contractility. The rise in intracellular Na^+ may also increase NCX reverse mode promoting Ca^{2+} influx (Despa *et al.* 2002*a*; Despa & Bers, 2013). Although moderate Ca^{2+} influx could potentially improve contractility linked to heart failure, Ca^{2+} overload may contribute to arrhythmias and cell death.

Because of these important roles of NCX, modulation of NCX function in heart failure has been suggested as a therapeutic target (Sipido *et al.* 2002; Pogwizd, 2003; Lee *et al.* 2005). For example, specific inhibitors for NCX could represent an alternative strategy in arrhythmia therapy (Antoons *et al.* 2012). However, pharmacological manipulation of NCX is problematic due to the lack of specific blockers. KB-R7943, long thought to be NCX specific, is in fact quite non-specific and seems to block other ionic currents (Reuter *et al.* 2002; Barrientos *et al.* 2009). Newer compounds such as SEA-0400 and SN-6 are more specific over other ion currents and more potent (Iwamoto *et al.* 2007; Niu *et al.* 2007). A unique and potentially desirable property of such blockers is their apparent preferential inhibition of NCX transport in the Ca^{2+} entry (reverse) mode, as initially proposed for KB-R7943 but later debated (Soma *et al.* 2006; Iwamoto *et al.* 2007). It is likely that this apparent selectivity is due to the asymmetric nature of the protein (i.e. different sensitivity of NCX for Na^+ and Ca^{2+} at the opposite sides of the membrane) and the ionic conditions used to test these drugs. Under circumstances of high intracellular Na^+, where reverse mode NCX activity dominates, NCX may reside in a state more sensitive to drug-mediated inhibition, thereby, leading to an apparently more efficient block. It should also be noted, however, that in more physiological conditions SEA-0400 will inhibit both forward and reverse mode to an equal extent (Ozdemir *et al.* 2008).

The development of a more selective inhibitor for NCX has strong clinical relevance for its potential therapeutic effects. However, the lack of structural information about

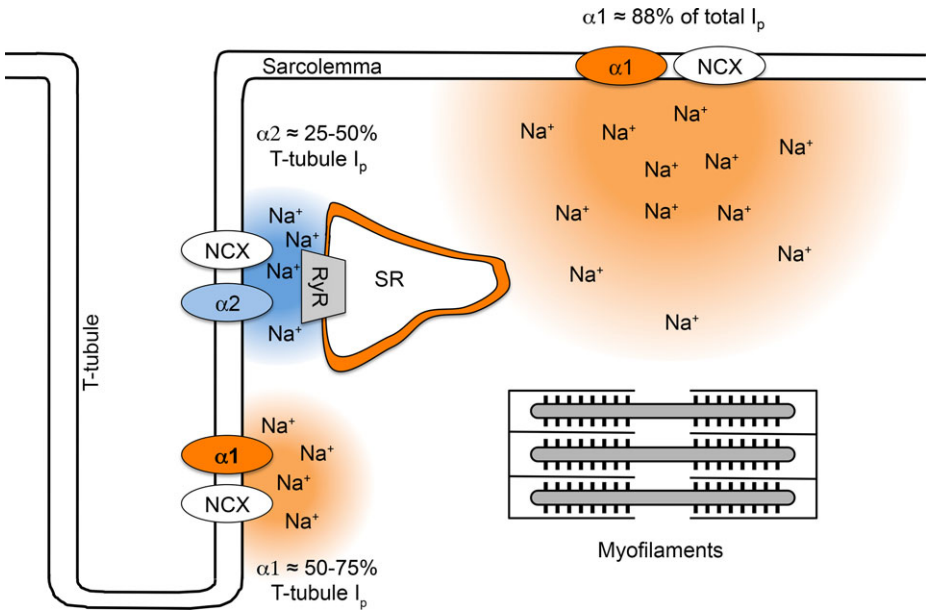

Figure 3. Diagram illustrating the relative distribution of Na^+ pump α1 and α2 subunits in a cardiac myocyte
The NKA resides and regulates intracellular Na^+ and Ca^{2+} (via NCX) in both external sarcolemma and T-tubular membranes. α1 is evenly distributed within the cells and is the dominant isoform providing around 88% of the total Na^+ pump current (I_p). α2 is 4–5 times more concentrated in the T-tubules compared to external sarcolemma and provides only around 12–24% of the total I_p but up to 50% of the I_p in the T-tubules. Thus, it has been suggested that α1 subunits may principally be involved in controlling bulk cytoplasmic Na^+. Functional evidence suggests that while both α1 and α2 subunits are expressed in T-tubular membranes, the possibility that the α2 subunit is relatively concentrated in the junctional areas (as shown above) remains to be structurally demonstrated. Estimates of pump current are based on the measurements of Berry *et al.* (2007).

the mammalian cardiac NCX is a major hindrance to achieving this goal and further studies are therefore essential to advance this field. Despite these limitations there have been a number of animal studies illustrating efficiency of NCX block in arrhythmias (Antoons *et al.* 2012). A certain lack of selectivity and reduction of L-type Ca^{2+} current (associated with NCX block) may be advantageous, as recently shown (Bourgonje *et al.* 2013).

Question or controversy: NCX and heart pathologies
At present there are no specific drugs for NCX. This prevents a full understanding of NCX's role in normal and pathophysiological conditions.
Additional structural information could help in developing new drugs. Can a selective drug block Ca^{2+} entry while maintaining Ca^{2+} extrusion?
What post-translational modifications does NCX undergo in pathological conditions that could alter its mode of operation?
Is activation or blockade of NCX likely to be of benefit in the treatment of heart pathologies?

The Na^+/K^+ pump – structure, function and regulation

In the session on 'Na/K pump – structure, function and regulation' Julie Bossuyt, Jerry Lingrel, Michael Shattock, Mordecai Blaustein, Jack Kaplan and Zi-Jian Xie were speakers, discussion leaders and panelists. Below is a summary of the materials presented and discussed in the session.

While the Na^+/Ca^{2+} exchanger is quantitatively one of the main Na^+ influx mechanisms in cardiac myocytes it is just one of a plethora of membrane transporters that utilize the energy in the trans-sarcolemmal Na^+ gradient to move ions, substrates, amino acids and metabolites into or out of the cell. All of these transport mechanisms dissipate the Na^+ gradient. Thus, the constant activity of the Na^+/K^+-ATPase (NKA, or Na^+ pump) is essential for re-establishing and maintaining this gradient. In cardiac and vascular smooth muscle the principal isoforms of the NKA are $\alpha1$ and $\alpha2$ and their physiological role is controlled both by their unique and independent signalling pathways, and their discrete subcellular distribution.

In cardiac muscle, the $\alpha2$ subunit has been suggested to play a specific subcellular role in preferentially regulating Na^+ in the dyadic cleft while the $\alpha1$ subunit plays more of a 'housekeeping' role regulating bulk cytoplasmic Na^+ (Dostanic *et al.* 2005). In vascular smooth muscle and skeletal muscle evidence also implicates the $\alpha2$ subunit in preferentially influencing contractile function (Juhaszova & Blaustein, 1997; Zhang *et al.* 2005; Radzyukevich *et al.* 2009). In the heart both isoforms ($\alpha1$ and $\alpha2$) associate with, and are regulated by, the FXYD protein phospholemman (PLM), with unphosphorylated PLM exerting a tonic inhibition on ion pumping (Pavlovic *et al.* 2007; Bossuyt *et al.* 2009). When PLM is phosphorylated (principally at Ser 63 and 68 by protein kinase C (PKC) or at Ser 68 by protein kinase A (PKA)) this inhibition is relieved and/or the pump is stimulated (Fuller *et al.* 2004; Despa *et al.* 2005). In this review we focus on recent advances in the regulation of NKA by PLM and studies elucidating the specific role of the $\alpha2$ isoform in cardiac,

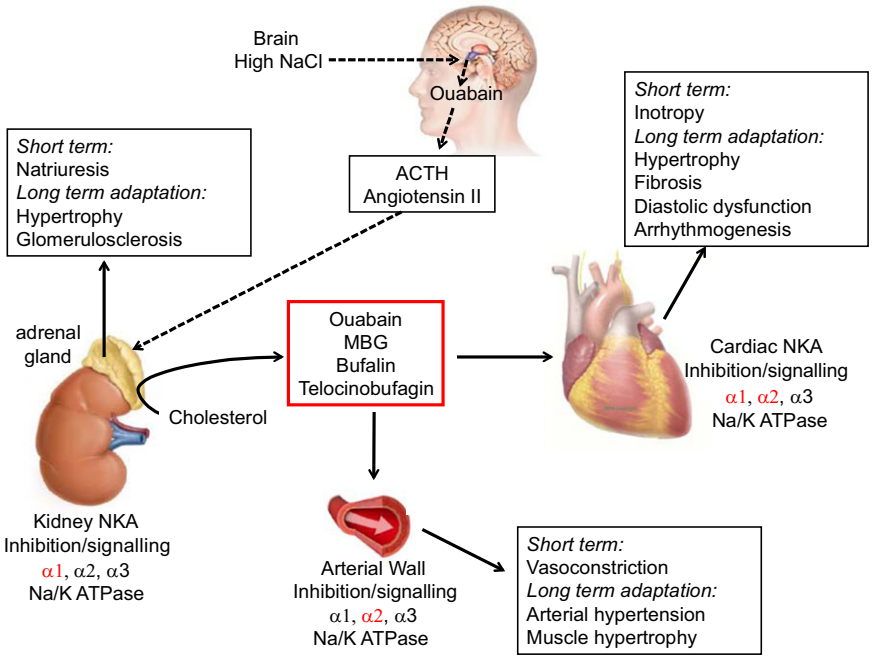

Figure 4. Proposed biosynthesis and function of endogenous cardiotonic steroids
Synthesis of endogenous cardiotonic steroids occurs in the adrenal cortex from cholesterol and is proposed to be under the control of adrenocorticotropic hormone (ACTH) and angiotensin II. These are produced in response to high salt and the resultant ouabain synthesis in the hypothalamus. Acute effects of endogenous cardiotonic steroids are mediated via inhibition of the NKA and lead to increased cardiac output, Na^+ excretion and vasoconstriction (NKA isoform-specific effects in each tissue are shown in red). Chronic exposure to cardiotonic steroids leads to adaptational remodelling changes of the affected tissue, possibly resulting in hypertrophy, fibrosis, hypertension and arrhythmogenesis. MBG, marinobufagenin. Adapted from Pavlovic (2014).

vascular and skeletal muscle, and the role of the NKA in general in cardiovascular disease.

Molecular interactions between PLM and NKA. *Stoichiometry considerations.* Several NKA crystal structures have been resolved now, greatly advancing our understanding of pump function and molecular interactions of the pump complex (Morth *et al.* 2007; Ogawa *et al.* 2009; Shinoda *et al.* 2009; Toyoshima *et al.* 2011; Kanai *et al.* 2013). In all the crystal structures to date, the $\alpha\beta$ complex, the minimal functional unit, was associated with a tissue-specific, regulatory FXYD protein (Kaplan, 2002; Jorgensen *et al.* 2003). Recently, a pool of 'pump-free' PLM multimers was identified in cardiac ventricle suggesting PLM expression is abundant enough to saturate $\alpha\beta$ complexes (Wypijewski *et al.* 2013). PLM oligomerization, specifically tetramerization, is further supported by structural and fluorescence studies in recombinant systems (Beevers & Kukol, 2006; Bossuyt *et al.* 2006; Song *et al.* 2011). These PLM oligomers could represent an inactive storage pool of PLM or may have non-pump-related functions (e.g. form channels) but fluorescence resonance energy transfer (FRET) data suggest that, like for the phospholamban–sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) system, the PLM–PLM and PLM–NKA equilibria may be dynamically linked (Bossuyt *et al.* 2006; Song *et al.* 2011). That is, PLM phosphorylation shifts the PLM–NKA equilibrium towards oligomers thereby relieving inhibition of the pump and vice versa.

The Na^+ pump as an $\alpha\beta$FXYD unit ($\alpha\beta$PLM for cardiac NKA) with a 1:1:1 stoichiometry appears to be the current consensus. Nonetheless, multiple studies have claimed that NKA can exist as an oligomer of multiple units ($(\alpha\beta\text{FXYD})^n$) in native membranes (reviewed by Taniguchi *et al.* 2001), but this idea remains highly controversial and it is unknown whether NKA oligomerization occurs in cardiac myocytes. Also, the Kaplan group recently showed that multiple β subunits can associate with α in the pump complex, suggesting that NKA subunit composition may also not be so rigid (Clifford & Kaplan, 2008, 2009). Finally, it is not known whether other FXYD proteins are expressed in cardiac myocytes at baseline or under stress (e.g. FXYD5 was identified in cardiac homogenates; Lubarski *et al.* 2005).

NKA–PLM interactions. Physical interaction between PLM and the NKA α1 and α2 isoforms was shown via co-immunoprecipitation (Crambert *et al.* 2002; Fuller *et al.* 2004; Bossuyt *et al.* 2005; Silverman *et al.* 2005) and crosslinking (Lindzen *et al.* 2006) in heterologous expression systems and cardiac myocytes. Crosslinking (Lindzen *et al.* 2006) and SERCA homology predictions (Sweadner & Donnet, 2001; Li *et al.* 2004) suggest that the transmembrane domain (TM) of FXYDs could reside in a groove formed by TM2, TM6 and TM9 of the NKA α subunit. However, crystal structures show FXYD proteins to be in proximity to, but not inside, this groove. The latter may be only one of many physiological conformations but mutational analysis showed that the E960 residue on NKA TM9 and the F28 residue on PLM are critical for both the functional effects on the pump and NKA–PLM FRET (Khafaga *et al.* 2012). The co-immunoprecipitation was not completely abolished by these mutations indicating that additional PLM–NKA interaction sites contribute to the robust physical association of PLM with NKA. A similar approach showed that PLM phosphorylation dramatically decreased NKA–PLM FRET without eliminating co-immunoprecipitation (Bossuyt *et al.* 2006). This is consistent with NMR studies where PLM phosphorylation increases the mobility of the cytosolic tail which is normally tightly associated with the negatively charged plasmamembrane (Franzin *et al.* 2007*a*,*b*; Teriete *et al.* 2007). Thus, PLM phosphorylation alters the PLM–NKA interaction but does not result in a complete dissociation (as has been proposed for phospholamban Asahi *et al.* 2001; Karim *et al.* 2006).

Question or controversy: stoichiometry interactions
Recent findings have called into question the dogma of the Na^+ pump complex as a functional heterodimer of α and β subunits that can associate with tissue-specific regulatory FXYD proteins.
Is the composition of the pump complex controlled via dynamic regulation of subunit expression or membrane abundance? Varying the composition would have functional consequences but to what extent does fine-tuning of the complex constituents occur? Or does this only become relevant in pathological situations such as heart failure?
If excess PLM is present in myocytes, possibly as PLM multimers, then what is their function?
Conversely, do 'alternative' complexes such as $\alpha\beta^n$ or $(\alpha\beta)^n$ represent the pool of NKA with alternative functional roles (e.g. signalling, adhesion)?

Differential roles of α1 and α2 in cardiac EC coupling. *Where are Na^+ pump isoforms located?* Of the four known NKA isoforms only α1–3 are expressed in cardiac muscle and their expression varies between species (Hensley *et al.* 1992; Sweadner *et al.* 1994; McDonough *et al.* 1996). The α1 isoform is both ubiquitous and quantitatively dominant, with the majority of species expressing significantly less, but functionally important, amounts of α2. On a subcellular level, the α2 isoform appears to be preferentially concentrated in T-tubular membranes while the α1 isoform is relatively uniformly distributed between T-tubular and external sarcolemmal membranes (Berry *et al.* 2007; Despa & Bers, 2007; Swift

et al. 2007; Bossuyt *et al.* 2009). It is worth noting that the α1 subunit remains quantitatively comparable and even the dominant isoform within the T-tubular membrane (with α1:α2 density ratios reported as 1:1 (rat), Despa & Bers, 2007, 3:2 (rat), Swift *et al.* 2007, and 4:1 (mouse) (Berry *et al.* 2007); however, the disproportionate functional impact of the α2 isoform suggests α2 may be further concentrated in the junctional regions of the T-tubule – this has yet to be investigated (Fig. 3). This preferential localization of the α2 subunit close to the junctional SR has also been reported in both smooth (*Juhaszova & Blaustein*, 1997) and skeletal muscle (Lavoie *et al.* 1997).

Is there a preferential role for the α2 subunit in cardiac EC coupling? The relative concentration of α2 in both cardiac and smooth muscle in the specialized membranes close to other ion translocators and the EC coupling machinery, has led to the proposal that the α2 subunit preferentially regulates the Na^+ (and hence Ca^{2+}) concentration in a limited (junctional SR/T-tubular) subcellular compartment that then strongly influences contraction. In this model, the α1 subunit is proposed to play more of a 'housekeeping' role regulating Na^+ in the bulk cytoplasmic compartment. Mice heterozygous for the α2 isoform ($\alpha2^{(+/-)}$) have a hyper-contractile phenotype (James *et al.* 1999), suggesting that Na^+/K^+ pumps containing the α2 subunit are functionally linked to the NCX. Further evidence for this functional compartmentation was provided by Dostanic *et al.* (2005) and Despa *et al.* (2012) who compared the effects of low-dose ouabain in wild-type hearts (expressing the ouabain-sensitive α2 isoform) with that in hearts expressing a genetically modified ouabain-resistant α2 isoform ('SWAP' mice). Dostanic *et al.*, using SWAP mice, also demonstrated that the α1 isoform is physically and functionally associated with the Na^+/Ca^{2+} exchanger and can also mediate inotropic responses to low-dose ouabain (Dostanic *et al.* 2004).

Question or controversy: EC coupling
While there is little doubt that the α2 isoform is important in the heart, a detailed understanding of its role is still missing.
Is there a preferential role for the NKA α2 subunit in EC coupling?
Within the T-tubule itself, are α2 isoforms further concentrated in regions close to the junctional SR?

Differential regulation of cardiac α1 and α2. The distinct distribution patterns of α1 and α2 suggest that the respective isoforms would be exposed to different signalling microdomains. Indeed, an early study in guinea pig ventricular myocytes found an exclusive link between PKA effects and NKA α1 isoforms, whereas PKC effects were targeted to α2 isoforms (Gao *et al.* 1999). Subsequent reports, however, found less divergent effects. Studies in both *Xenopus* oocytes and SWAP mice found that PKA activation increases the apparent Na^+ affinity of both α1 and α2 (Bibert *et al.* 2008; Bossuyt *et al.* 2009), but in detubulated mouse myocytes β-adrenergic effects were limited to the α1 isoforms (Berry *et al.* 2007). FRET experiments in cells from the human embryonic kidney (HEK) cell line found that submaximal PKA activation had less effect on the PLM–NKA α2 isoform interaction than on the α1 isoform, indicating that the intrinsic sensitivity to PKA regulation is slightly different for the two isoforms (Bossuyt *et al.* 2009). The PKC effects on NKA are more complex. Even at the level of the overall effect there are some discrepancies: most agree that PKC stimulates NKA (Wang *et al.* 1998; Gao *et al.* 1999; Han *et al.* 2006; Bibert *et al.* 2008; Fuller *et al.* 2009); however, inhibition was also reported (Lundmark *et al.* 1999; Buhagiar *et al.* 2001; White *et al.* 2009). Some of these differences could be due to which PKC isoform is engaged or the intracellular Ca^{2+} level used in the experiment (since Ca^{2+} can modulate PKC effects on NKA). The mechanism of the increased pump activity also remains unclear as changes in V_{max} alone (Han *et al.* 2006; Bibert *et al.* 2008) or both V_{max} and Na^+ affinity (Bossuyt *et al.* 2009) were reported. These mechanistic differences could be due to differential phosphorylation of PLM by PKA and PKC (Han *et al.* 2006; Fuller *et al.* 2009). In oocytes, PKC also affected only the turnover rate of the α2 isoforms (Bibert *et al.* 2008). In SWAP mice on the other hand, PKC increased the Na^+ affinities (via phospholemman) of both isoforms while only increasing the V_{max} for the α2 isoform (Bossuyt *et al.* 2009). Interestingly, in contrast to PKA, PKC similarly affected PLM–NKA FRET for both NKA isoforms. Thus, despite recent advances, the differential regulation of NKA isoforms by protein kinases has not been clearly established.

In addition to phosphorylation, other post-translational modifications, such as glutathionylation and palmitoylation of cysteine residues within the pump complex, can regulate NKA function (Figtree *et al.* 2009; Howie *et al.* 2013). Oxidant stress and modification of NKA thiols has long been known to inhibit the NKA activity (Shattock & Matsuura, 1993; Haddock *et al.* 1995) and, more recently, White *et al.* have shown that angiotensin II-mediated oxidant stress can inhibit Na^+/K^+ pump activity via a PKC and nitric oxide synthase-dependent mechanism (White *et al.* 2009). While the α, β and PLM subunits of NKA have all been reported to be palmitoylated (Howie *et al.* 2013), and the β subunit preferentially glutathionylated (Figtree *et al.* 2009), at present whether regulation via such thiol modification differs between α subunit isoforms is unknown. Interestingly, a recent report from Gao *et al.* shows that transmural gradients of angiotensin II may

differentially regulate Na^+/K^+ pump function in response to mechanical load (Gao *et al.* 2014).

The Na^+ pump in hypertrophy and heart failure. *Evidence and significance of Na^+ dysregulation.* In most mammalian hearts with a long action potential, intracellular Na^+ ($[Na^+]_i$)is maintained at around 4–8 mM (Harrison *et al.* 1992; Despa *et al.* 2002*a*). In mice and rats, the combination of a high heart rate and short action potential is associated with a higher Na^+ of around 10–20 mM (Shattock & Bers, 1989; Despa *et al.* 2002*a*). As discussed earlier, the maintenance of this large trans-sarcolemmal inward gradient is essential for numerous transport and electrogenic processes and its dissipation in various pathologies such as ischaemia/reperfusion (Tani & Neely, 1989; Neubauer *et al.* 1992), hypertrophy or heart failure (Pogwizd *et al.* 2003; Verdonck, 2003) is clearly detrimental. In hypertrophy and heart failure many aspects of EC coupling are altered; however, in combination with these the elevation in intracellular Na^+ may contribute to the negative force–frequency relationship, slowed relaxation and arrhythmias (Pieske, 2002). Recently, O'Rourke and colleagues have also shown that elevation of cytoplasmic Na^+ can impair mitochondrial energetics (Liu & O'Rourke, 2008; Kohlhaas *et al.* 2010; Liu *et al.* 2010). Mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) plays a key role in linking ATP production to ATP demand (i.e. mechanical activity) and as Ca^{2+} rises in the cell, so does $[Ca^{2+}]_m$; this activates mitochondrial enzymes to step-up ATP production (Liu & O'Rourke, 2008; Kohlhaas *et al.* 2010). This relationship, which crucially matches ATP supply to demand, is blocked when cytosolic Na^+ is elevated (Liu *et al.* 2010). The rise in Na^+ activates Na^+/Ca^{2+} exchange in the inner mitochondrial membrane and this keeps $[Ca^{2+}]_m$ low preventing ATP supply meeting demand, leaving the heart metabolically compromised. Not only might this contribute to the known metabolic insufficiency in failing hearts but Kohlhaas *et al.* have shown that this mechanism increases mitochondrial free radical formation in failing hearts, further exacerbating injury (Kohlhaas *et al.* 2010).

While a component of the elevation of $[Na^+]_i$ may reflect an increase in Na^+ influx (Despa *et al.* 2002*b*), there is a large body of evidence showing that Na^+/K^+ pump function may also be compromised (Pogwizd *et al.* 2003; Verdonck *et al.* 2003*a*,*b*; Boguslavskyi *et al.* 2014*b*). Specifically in cardiac hypertrophy many studies have shown that Na^+/K^+ pump function, and/or expression, is reduced (Pogwizd *et al.* 2003; Verdonck *et al.* 2003*a*,*b*; Bossuyt *et al.* 2005; Boguslavskyi *et al.* 2014*b*).

Role of PLM in hypertrophy and failure. Despite the importance of PLM in regulating intracellular Na^+, and the considerable evidence suggesting that intracellular Na^+ is elevated in hypertrophied and failing myocardium, and contributes to diastolic dysfunction, the role of PLM and its phosphorylation in the overloaded and failing heart has not been systematically characterized. Bossuyt *et al.* have reported hyper-phosphorylation of PLM in a rabbit model of volume overload-induced dilated failure (Bossuyt *et al.* 2005) while El-Armouche *et al.* (2011) have reported hypo-phosphorylation of PLM in failing human hearts, attributable to down-regulation of Inhibitor-1 and increased PP-1 phosphatase activity. Recently, Boguslavskyi *et al.* (2014*b*) and Correll *et al.* (2014) have reported a reduction in both PLM expression and phosphorylation in mouse models of hypertrophy that, in the Boguslavskyi study, has been directly related to declining Na^+/K^+ pump function and intracellular Na^+ elevation.

The lack of consensus regarding the PLM phosphorylation status may reflect different stages of the disease process or different animal models. Simply reporting hypo- or hyper-phosphorylation of PLM also does not distinguish between an effect that causally influences the progression of the disease from one that is simply a bystander – reporting dynamic disease-induced changes in PKA and PKC signalling pathways. In order to test this, Boguslavskyi *et al.* have used a mutant PLM^{3SA} knock-in mouse (in which PLM has been rendered unphosphorylatable) (Boguslavskyi *et al.* 2014*b*). They showed that preventing PLM phosphorylation in mice subjected to aortic constriction further inhibits NKA function, increases cellular Na^+ overload and exacerbates adverse hypertrophic remodelling. This suggests that in heart failure, PLM hypo-phosphorylation in mice is not simply a bystander effect (reporting dynamic disease-induced changes in PKA and PKC signalling) but is causally involved in disease progression.

Role of the α2 subunit in hypertrophy and failure. The α2 subunit has been suggested to not only play a preferential role in normal EC coupling but also in the pathological response to cardiac hypertrophy and heart failure. In a recent study, Correll *et al.* have reported that the over-expression of α2 (but not α1) preferentially protects against adverse remodelling in the hypertrophic heart and improves Ca^{2+} and Na^+ handling. In this study, the 'forced' over-expression of α2 leads to a reduction in expression and phosphorylation of PLM and an overall increase in the Na^+ sensitivity of the NKA. They suggest that the α2 subunit is 'less regulated by PLM', thus providing a mechanism by which α2 can efficiently couple to NCX to maintain ion (and particularly Ca^{2+}) regulation. Adaptive increases in α2 expression are also seen in mice expressing an unphosphorylatable form of PLM (PLM^{3SA}), perhaps allowing the normalization of EC coupling despite an increase in the bulk cytoplasmic Na^+ concentration (Boguslavskyi *et al.* 2014*b*).

Since cardiac remodelling and dysfunction in heart failure are exacerbated by preventing PLM phosphorylation (Boguslavskyi *et al.* 2014*b*), and $\alpha 2$ overexpression is protective (Correll *et al.* 2014), then this raises the interesting possibility that Na^+/K^+ pump stimulation may be therapeutically useful in heart failure (Rasmussen & Figtree, 2007; Shattock, 2009) and, in particular, isoform-selective $\alpha 2$ stimulation. Superficially, this seems to fly in the face of 200 or more years of digitalis use to treat heart failure. However, it would not be the first time that counter-intuitive therapeutics prove beneficial as demonstrated by the historic aversion to negative inotropes in heart failure and the remarkable recent success of β blockers. In fact, digitalis seems to only provide symptomatic relief and, in the largest trial of its kind, the Digitalis Investigation Group (DIG) concluded that digoxin reduced hospitalization due to worsening heart failure symptoms but had no long-term effect on mortality (Hobbs, 1997). This issue is, however, undoubtedly more complex, not least because of the presence of endogenous ouabains in heart failure patients and the known ouabain–digoxin antagonism (see Blaustein, 2014 for review).

Question or controversy: heart failure
What do we need for the treatment of heart failure – Na^+/K^+ pump inhibitors or stimulators?
The preferential role of the $\alpha 2$ subunit in EC coupling may mean that an isoform-specific *inhibitor* might be able to induce an ionotropic benefit WITHOUT triggering Ca^{2+} overload and arrhythmias. Is it possible to achieve an increase in SR Ca^{2+} load and positive inotropy WITHOUT risking Ca^{2+} overload?
Conversely, it could be argued that the protective role of the $\alpha 2$ subunit in heart failure suggests that an isoform-specific ($\alpha 2$) *stimulator* might prevent Na^+ and Ca^{2+} overload and limit adverse remodelling.

Isoform-specific roles in control of blood pressure. The control of blood pressure, and specifically the aetiology of primary hypertension is now recognized to have both peripheral and central components. There is mounting evidence that the misregulation of the NKA may contribute to both.

Central role of $\alpha 2$ in regulation of blood pressure. While primary hypertension involves the interplay of genetic and environmental factors, such as high-salt intake, obesity, insulin resistance, low physical activity levels and stress, there is now increasing evidence that elements of such hypertension may be neurogenic in origin (Huang *et al.* 2006; Esler *et al.* 2010). A number of studies have shown that the level of Na^+ in the cerebrospinal fluid (CSF) is a critical factor contributing to the pressor response induced by elevated dietary Na^+ (Huang *et al.* 1998, 2001, 2004; Van Huysse *et al.* 2011). Van Huysse *et al.* (2011) have shown that the rise in blood pressure in response to CSF Na^+ elevation is mediated by the central release of an endogenous ouabain-like substance that specifically targets the ouabain-sensitive $\alpha 2$ subunit of the NKA. The cell types and CNS pathways involved in this signalling pathway have yet to be identified. However, in the brain the $\alpha 2$ NKA isoform is concentrated in the glia where, coincidentally, 90% of brain angiotensinogen is also found (Schinke *et al.* 1999). Since, upregulation of the glial renin–angiotensin system specifically raises blood pressure (Morimoto *et al.* 2001, 2002), this cell type may be central in the pressor response to Na^+ load.

Question or controversy: blood pressure control – central role of NKA
What is/are the cellular sources and identities of the endogenous ouabain-like substances that mediate the pressor response to Na^+ elevation in CSF?
In human hypertension, does this pathway preferentially target the $\alpha 2$ NKA isoform in the CNS as it does in mice?
Does this offer a novel therapeutic target for the treatment of primary hypertension?

Peripheral role of $\alpha 2$ in the regulation of blood pressure. In addition to a central role for the NKA in blood pressure control, the activity of the Na^+/K^+ pump can also locally regulate both endothelial and vascular smooth muscle (VSM) function. The NKA in VSM, as in myocardial cells, sets and maintains the Na^+ and K^+ electrochemical gradient and, in this way, modulates the activity of other membrane ion channels and signalling pathways (Shelly *et al.* 2004; Zhang *et al.* 2005). The relatively high input impedance of VSM cells also means that the electrogenic pump can theoretically directly and substantially modulate membrane potential (and hence Ca^{2+} influx) with activation of the pump causing hyperpolarization and relaxation, and inhibition causing depolarization and vasoconstriction (Casteels *et al.* 1977; Quinn *et al.* 2000; Burns *et al.* 2004; Molin *et al.* 2005). However, the extent to which electrogenic NKA activity in vascular smooth muscle contributes to membrane potential varies between experimental preparation and vascular beds. Selective $\alpha 2$ inhibition by low-dose ouabain also can mediate vasoconstriction in the absence of changes in membrane potential. Thus, while NKA activity can undoubtedly modulate vascular tone, in many studies it is difficult to dissociate effects arising from NKA-induced changes in membrane potential from those induced by changes in ion channels or secondary to changes in local ion gradients, NCX activity etc. The situation is further complicated

by studies showing that the effects of NKA inhibition or stimulation differ between intact vessels and those in which the endothelium has been denuded – suggesting that both smooth muscle and endothelial NKA activity can modulate vascular tone in intact tissues (see below).

As in other tissues the vascular NKA is expressed as a heterodimer composed of a catalytic α subunit and a glycosylated β subunit (Lingrel & Kuntzweiler, 1994). Of the four known α isoforms (α1–4) only α1 and α2 were found in VSM cells (Shelly *et al.* 2004; Pritchard *et al.* 2010). These isoforms have different cellular distribution, expression and functional significance. In VSM cells the α1 isoform is by far the most abundant and is thought to play a 'house-keeping' role controlling bulk cytoplasmic Na^+ (Juhaszova & Blaustein, 1997; Weston *et al.* 2002). In rodents, this α1 isoform is relatively insensitive to ouabain. The α2 ouabain-sensitive isoform, while much less abundant, has a highly localized cellular distribution and is suggested to play a role in regulating Na^+ in microdomains with preferential access to other membrane transporters (*Juhaszova & Blaustein*, 1997; Shelly *et al.* 2004; Pritchard *et al.* 2010). The close association between the α2 subunit, NCX and the intracellular SR membrane has been suggested to give the α2 subunit a preferential role in regulating Ca^{2+} (and hence constriction) in VSM cells (*Juhaszova & Blaustein*, 1997; Arnon *et al.* 2000; Lee *et al.* 2006; Linde *et al.* 2012). Recently it has been shown that NKA and Ca^{2+}-handling proteins form a functionally coupled unit where the concentration of Na^+ in a subcellular microdomain modulates submembrane Ca^{2+} and hence SR Ca^{2+} load (Matchkov *et al.* 2007; Pritchard *et al.* 2010). Reduction of NKA activity (specifically of α2) by pharmacological inhibition or transgenesis leads to enhanced agonist-induced vascular contractility and myogenic tone (Zhang *et al.* 2005). Matchkov *et al.* showed that α2, through modulation of local Ca^{2+}, is very important in gap junction coupling between VSM cells and hence affects vasomotion and the spread of vasoconstriction and relaxation (Matchkov *et al.* 2007, 2012). There is also a large body of evidence showing that NKA is important for NO- and endothelium-derived hyperpolarizing factor (EDHF)-dependent responses (Gupta *et al.* 1994; Sathishkumar *et al.* 2005; Leung *et al.* 2006; Dora *et al.* 2008; Matchkov *et al.* 2012). Furthermore, the α2 subunit is present at myo-endothelial junctions where local increases of K^+ concentration (due to the opening of endothelial Ca^{2+}-activated K^+ channels) can activate the Na^+/K^+ pump and induce VSM cell hyperpolarization and relaxation (Dora *et al.* 2008).

Given the involvement of NKA (in particular α2) in the regulation of myogenic tone, vasomotion, cell-to-cell coupling, and NO- and EDHF-dependent relaxation it is perhaps not surprising that NKA activity may be an important modulator of blood pressure (BP) *in vivo*. Circulating endogenous ouabain, for example, has been shown to be involved in hypertension in both animals and humans (see below) (Manunta *et al.* 1999; Schoner, 2002). Furthermore, in mice expressing ouabain-resistant α2 NKA, Dostanic *et al.* showed that ouabain-induced hypertension is mediated by the α2 subunit (Dostanic *et al.* 2005). Further experiments with these mice revealed the importance of ouabain-binding sites on NKA in ACTH-induced hypertension (Dostanic-Larson *et al.* 2005; Lorenz *et al.* 2008). Results obtained from other genetic models with global down-regulation or over-expression of NKA demonstrate that the activity of NKA has a major impact on BP (Zhang *et al.* 2005; Pritchard *et al.* 2007). Interestingly, in mice with cardiovascular-specific knockout of α2, basal BP was not affected, but the hypertensive response to ACTH treatment was blunted (Rindler *et al.* 2011).

In the vasculature, as in cardiac and skeletal muscle, NKA is associated with FXYD1 (phospholemman) (Palmer *et al.* 1991; Rembold *et al.* 2005). Dey *et al.* demonstrated that PLM phosphorylation by PKC activates the α2 isoform pulmonary artery smooth muscle cells (Dey *et al.* 2012). Recently, Boguslavskyi *et al.* have shown that PLM phosphorylation is critically important in modulating the constrictor response of aortic smooth muscle to phenylephrine and the relaxing effects of an NO donor (Boguslavskyi *et al.* 2014*a*). In the heart, Pavlovic *et al.* have shown that NO stimulates NKA via PKC-mediated PLM phosphorylation (Pavlovic *et al.* 2013). Whether similar pathways exist in the vasculature is yet to be determined.

In summary, direct (endogenous cardiotonic steroids, local K^+) or indirect (PLM dependent) regulation of vascular NKA activity affects myogenic tone through altering Na^+ and Ca^{2+} homeostasis and/or stimulating Src-dependent signalling (see below) which significantly influences blood pressure *in vivo*.

Question or controversy: blood pressure control – vascular smooth muscle

Does the NKA (and specifically the α2 subunit) play a role in regulating vascular tone and if so, are these effects mediated by electrogenic NKA transport and changes in membrane potential or by changes in transmembrane ionic concentrations?

What are the relative roles of smooth muscle *vs.* endothelial NKA in regulating tone?

Is vascular smooth muscle NKA regulated by phospholemman and, if so, via which signalling pathways?

Is this signalling–PLM–NKA–tone pathway defective in hypertension and/or does it provide a therapeutic target for the treatment of hypertension?

Na^+/K^+ pump function and skeletal muscle. *Skeletal muscle fatigue.* While many other mechanisms (such as P_i accumulation, acidosis, ATP depletion, generation of reactive oxygen species etc.) have been implicated in fatigue (see Allen *et al.* 2008 for review), there is substantial evidence that extracellular K^+ accumulation (and the associated disruption of intracellular Na^+ and K^+ concentrations) is central to this process (Medbo & Sejersted, 1990; Juel *et al.* 2000; Clausen, 2003; Allen *et al.* 2008). The NKA plays a central role in limiting extracellular K^+ accumulation during exercise (Clausen, 2003). Interventions that stimulate the pump (such as the β_2-adrenoceptor agonist salbutamol) can decrease K^+ accumulation and limit fatigue (Clausen, 2003), while interventions that inhibit the pump (such as ouabain) increase K^+ accumulation and exacerbate fatigue (Nielsen & Clausen, 1996). It is now widely accepted that the increased activity of the NKA during exercise 'protects' skeletal muscle against fatigue during exercise (Juel *et al.* 2000; Clausen, 2003). One of the strongest drivers for activation of the Na^+/K^+ pump is the elevation of intracellular Na^+. However, it is also clear that sympathetic stimulation (which inevitably accompanies exercise *in vivo*) also directly activates the pump and can increase its basal turn-over by up to 20-fold (Allen *et al.* 2008). This appears to be particularly important when extracellular K^+ is elevated (Nielsen & de Paoli, 2007). An increasing number of studies have implicated PLM as the link between cell signalling and exercise-induced NKA stimulation (Thomassen *et al.* 2010, 2011, 2013; Benziane *et al.* 2011). PLM phosphorylation is increased by exercise via a PKCα-dependent mechanism (Thomassen *et al.* 2011) possibly in response to intracellular Ca^{2+} elevation, although the exact signalling pathways involved have yet to be determined.

Role of the α2 subunit. While quantitatively the α1 subunit dominates cardiac muscle, in skeletal muscle up to 87% of total NKA expression is estimated to be α2 (He *et al.* 2001). As in the heart, α2 in skeletal muscle appears to preferentially play a key role in regulation of contraction and protection against fatigue. Radzyukevich *et al.* (2013) used a skeletal muscle-specific α2 knockout to demonstrate that α2, but not α1, is responsible for acute upregulation of NKA activity and protects skeletal muscle against fatigue (Radzyukevich *et al.* 2013). This and other studies also show that the functional α2 subunit is preferentially located in the T-tubules and it is potassium regulation within the T-tubular lumen that may be critical in determining fatigue (Lavoie *et al.* 1997; Williams *et al.* 2001; Heiny *et al.* 2010). A large fraction of skeletal muscle α2 may also be resident in submembrane vesicles readily available for trafficking to and from the plasma membrane in response to receptor stimulation or demand (Hundal *et al.* 1992; Al-Khalili *et al.* 2003).

Question or controversy: skeletal muscle
Does the phosphorylation of phospholemman regulate skeletal muscle NKA and, if so, via which signalling pathways?
Does dysregulation of these pathways contribute to muscle fatigue?

Endogenous cardiotonic steroids. Realization that cardiotonic steroids (CTS) are present in nanomolar concentrations in the serum of experimental animals and humans has intensified research into their physiological and pathophysiological roles over the last 20 years (Hamlyn *et al.* 1982; Bagrov *et al.* 1998; Gallice *et al.* 1998; Gonick *et al.* 1998; Manunta *et al.* 1999; Harwood *et al.* 2001; Periyasamy *et al.* 2001; Komiyama *et al.* 2005; Kennedy *et al.* 2006; Fedorova *et al.* 2009; Li *et al.* 2010). Interestingly, a recent study using a highly sensitive UPLC–MS–MS technique failed to detect ouabain *per se* in human plasma suggesting that the CTS detected in other studies using radioimmunoassays represent compounds that are structurally similar, but not identical, to ouabain (Baecher *et al.* 2014). CTS are divided into two structurally distinct groups, cardenolides (ouabain-like compounds and digoxin) and bufadienolides (marinobufagenin, bufalin and telocinobufagin). Evidence is accumulating that most CTS are synthesized from cholesterol in the adrenal glands and possibly hypothalamus (Laredo *et al.* 1995; Dmitrieva *et al.* 2000; Murrell *et al.* 2005). The trigger for biosynthesis initiation is complex, with serum concentrations of CTS increasing in response to kidney dysfunction (Komiyama *et al.* 2005; Stella *et al.* 2008; Tian *et al.* 2010), volume expansion (Manunta *et al.* 2006), salt accumulation in the brain (Huang *et al.* 2004), adrenocorticotropic hormone, angiotensin II, vasopressin, and phenylephrine (Laredo *et al.* 1995; Shah *et al.* 1999). Growing evidence suggests that CTS secretion is involved in the pathogenesis of a number of diseases such as hypertension, pre-eclampsia and uraemic cardiomyopathy.

Cardiotonic steroids in blood pressure control. The mechanisms that govern CTS-mediated hypertension development have not yet been fully elucidated; however, evidence is accumulating that in response to high salt intake, a ouabain-like compound is secreted by the hypothalamus (Murrell *et al.* 2005), whereas both ouabain and marinobufagenin are secreted by the adrenal glands (Laredo *et al.* 1995; Dmitrieva *et al.* 2000) and that this leads to both central and peripheral blood pressure elevation (Fig. 4). Specifically, high salt intake elevates both plasma and cerebrospinal fluid Na^+ and this induces the secretion of CTS by the adrenals and the hypothalamus (Huang *et al.* 2004). Intracerebroventricular infusion of

Na^+-rich cerebrospinal fluid (Van Huysse *et al.* 2011) or ouabain (Huang *et al.* 2004) increased sympathetic nerve activity, heart rate and blood pressure in rodents and these effects were partially reversed by Digibind (anti-digoxin Fab® fragments). On the other hand, chronic intravenous infusion of ouabain or marinobufagenin at concentrations comparable with *in vivo* plasma levels lead to an increase in arterial pressure and cardiac hypertrophy (Pamnani *et al.* 1994; Ferrandi *et al.* 2004; Kennedy *et al.* 2006). Furthermore, hypertension induced either by salt loading, ouabain infusion or pre-eclampsia is reduced by immunoneutralization with anti-CTS antibodies (Fedorova *et al.* 2002, 2007) or CTS antagonists rostafuroxin (Ferrari *et al.* 2006) and resibufogenin (Horvat *et al.* 2010). It is important to emphasize that ouabain binds to the Digibind with high affinity, whereas marinobufagenin binding affinity is lower (Pullen *et al.* 2004) suggesting that a ouabain-like substance is the dominant CTS in blood pressure regulation. Whereas the effects of brain-derived endogenous ouabain require further characterization, plasma ouabain produced by the adrenal glands can contribute to acute vasoconstriction via inhibition of the $\alpha 2$ sodium pumps in the smooth muscle, as discussed in the previous section 'Isoform-specific roles in control of blood pressure'. Interestingly, 3 days of salt loading in healthy individuals did not result in a change in blood pressure (Manunta *et al.* 2006) suggesting that acute salt-loading in a healthy individual has no pathological consequences. However, it is clear that during chronic salt loading or chronic ouabain infusion, hypertension persists. It is likely that this is mediated via a combination of chronic elevation of brain Na^+, leading to increased sympathetic nerve activity, and the direct effects of endogenous ouabain on the smooth and cardiac muscle, potentially resulting in increased cardiac output, increased total peripheral resistance and thus elevation in blood pressure. Furthermore, reported CTS-mediated activation and internalization of the Na^+/K^+–Src–epidermal growth factor receptor (EGFR) complex (Liu *et al.* 2002, 2004, 2005) as well as up-regulated arterial expression of the NCX1, SERCA2 and TRPC6 (Blaustein *et al.* 2012; Pulina *et al.* 2013) could contribute to arterial remodelling of the affected tissue and thus leading to established hypertension. Whereas both ouabain and marinobufagenin have been implicated in the regulation of blood pressure and hypertension development, most of the currently available data imply that ouabain is the dominant CTS. Interestingly, digoxin has been shown to oppose ouabain-induced hypertension in rats (Kimura *et al.* 2000; Manunta *et al.* 2000) and possibly even in humans (Abarquez, 1967). Song *et al.* (2014) report that low doses of digoxin can antagonize the effects of ouabain, possibly explaining the recently reported beneficial effects of low doses of digoxin therapy in heart failure patients (Ahmed *et al.* 2006).

Question or controversy: endogenous cardiotonic steroids

What is the biological significance of the diversity of endogenous CTS?

Do individual CTS play specific roles in the development of cardiovascular dysfunction?

Can we exploit knowledge of the antagonism between CTS molecules to therapeutic advantage?

Cardiotonic steroids in chronic kidney disease. It has been evident for many years that patients with chronic kidney disease (CKD) and end-stage renal disease (ESRD) are at increased risk of developing cardiovascular disease (Tyralla & Amann, 2002; Best *et al.* 2004). Renal failure results in a cardiomyopathy characterized by left ventricular hypertrophy, reduced ejection fraction, myocardial fibrosis and arrhythmias (Tyralla & Amann, 2002; Best *et al.* 2004). Although the cause of cardiomyopathy development is likely to be multifactorial, McMahon and colleagues have observed elevated diastolic intracellular Ca^{2+} (due to compromised NCX activity) in the hearts of rats with impaired renal function (McMahon *et al.* 1996, 2002, 2006; Donohoe *et al.* 2000).

Considering that serum levels of ouabain, telocinobufagin and marinobufagenin are substantially elevated in patients with ESRD (Komiyama *et al.* 2005; Stella *et al.* 2008) and animals with CKD (Kennedy *et al.* 2006, 2008), it is reasonable to propose that CTS-mediated Na^+/K^+ inhibition and subsequent increase in intracellular Na^+ could be the mechanism responsible for the observed myocardial Ca^{2+} overload and could explain contractile dysfunction, negative force–frequency relationship, increased susceptibility to arrhythmias and left ventricular hypertrophy. Ca^{2+} overload in the smooth muscle could also lead to the development of hypertension as discussed in the previous section. In a rat model of CKD, Pavlovic *et al.* have shown that cardiac Na^+/K^+ pump is inhibited and that this inhibition is attenuated by the removal of CTS using DigiFAB® (Pavlovic, 2010). On the other hand, Liu and colleagues have proposed an alternative model of CTS-mediated cardiomyopathy development. This model proposes that a fraction of Na^+/K^+ pump subunits are localized in the caveolae and are not involved in transport of Na^+ and K^+ ions but instead act as receptors for CTS (Pierre & Xie, 2006). These 'inactive' pumps are physically associated with other key signalling proteins such as EGFR and Src (Wang *et al.* 2004; Liang *et al.* 2007). Binding of the CTS leads to activation of hypertrophic and fibrotic signalling cascades via Na^+/K^+–Src extracellular signal-regulated kinase (Li *et al.* 2010), independently of changes in intracellular Na^+ and Ca^{2+} (Liu *et al.* 2000). This alternative signalling pathway does not account for the reported Na^+/K^+ pump inhibition that can be reversed by anti-CTS antibodies

in patients with diabetes (Bagrov *et al.* 2005) or ESRD (Periyasamy *et al.* 2001), nor the accompanying increases in intracellular Na^+ and Ca^{2+} reported by other groups (Peng *et al.* 1996; Dong *et al.* 2004; Andrikopoulos *et al.* 2011). Na^+/K^+ pump inhibition via CTS alone can explain the cardiovascular dysfunction observed in CKD patients, whereas the signalling model can only account for the hypertrophy and hypertension development. Whether CTS induce hypertrophic growth in the heart via the ionic or signalling pathway remains to be resolved, however, the weight of evidence in support of both pathways makes it likely that they act in conjunction with each other.

Question or controversy: CKD-induced cardiomyopathy

Although the CTS binding site is highly conserved across the evolutionary spectrum (Lingrel, 2010), rats and mice possess an Asn122His substitution in their NKA α1 isoform, making it less sensitive to CTS (Price & Lingrel, 1988). Considering that most studies are conducted in rat and mouse, a question of relevance of some of the data to human physiology requires investigation.

The relative contribution of the CTS signalling *vs.* effects on ion transport of the NKA in CKD and hypertension have yet to be determined.

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Additional information

Competing interests

None declared.

Funding

The authors acknowledge grant support from the British Heart Foundation (RG/12/4/29426; to M.J.S.) and National Institutes of Health (P01-HL080101 to D.M.B.) and additional grants to other authors that allowed them to participate. Sponsors who subsidized conference costs can be found at the website below.

Acknowledgements

We thank the meeting organizers, Advisory Board and all conference participants for their contributions to the scientific exchange and constructive discussions. For conference information see https://basicscience.ucdmc.ucdavis.edu/ucd-cvs-2014/index.html.