The Pump, the Exchanger, and Endogenous Ouabain
Signaling Mechanisms That Link Salt Retention to Hypertension


The central roles of salt (NaCl) and the kidneys in the pathogenesis of most forms of hypertension are well established. The linkage between NaCl retention and blood pressure (BP) elevation is often referred to as “whole body autoregulation.” Surprisingly, however, the precise mechanisms that underlie this linkage (ie, the signaling pathway) have escaped elucidation. Here, we examined the evidence that endogenous ouabain (EO), Na+/pumps (Na,K-ATPase), and the Na/Ca exchanger (NCX) are critical molecular mechanisms in this pathway.

Ca²⁺ and the Control of Myogenic Tone
At constant cardiac output (CO), mean arterial BP = CO × TPR (where TPR is total peripheral vascular resistance). In most (chronic) hypertension, in humans and animals, the CO is relatively normal, and the high BP is maintained by an elevated TPR. TPR is controlled dynamically by vascular constriction/dilation in small “resistance” arteries, which exhibit tonic constriction (“tone”). This can be studied in isolated, cannulated small arteries that develop spontaneous (myogenic) tone (MT), under constant or increasing intraluminal pressure. Indeed, the level of tone in isolated arteries “is often comparable to that observed in the same vessels in vivo,” and may even be used to predict BP changes (see below).

MT is triggered by Ca²⁺ entry, primarily through voltage-gated Ca²⁺ channels in arterial smooth muscle (SM; ASM) cells, and contraction is activated by the rise in cytosolic Ca²⁺ concentration ([Ca²⁺]CYT). In NaCl-dependent hypertension, the enhanced vasoconstriction and increased tone and TPR are, at least in part, functional and reversible phenomena. Numerous mechanisms contribute to the regulation of myocyte [Ca²⁺]CYT and vasoconstriction, but the plasma membrane (PM) NCX provides an unique, direct link between Na⁺ and [Ca²⁺]CYT and, thus, Ca²⁺ signaling and contraction in ASM cells. NCX-mediated Ca²⁺ transport is governed by the Na⁺ electrochemical gradient generated by the PM Na⁺ pump.

We proposed that an endogenous Na⁺ pump inhibitor, ie, a ouabain-like compound, with vasotonic action might be secreted in response to NaCl retention. In other words, this substance might be a missing hormonal link between NaCl retention and the increased TPR and hypertension. Conservation of the high-affinity ouabain binding site amino acid sequence in mammalian evolution (see below) implies that there must be an endogenous ligand for this site. Partial Na⁺ pump inhibition by the endogenous inhibitor should promote the net gain of Ca²⁺ via the myocyte NCX and thereby augment Ca²⁺ signaling and vasoconstriction.

Endogenous Ouabain
These ideas triggered an intense international search for the postulated endogenous Na⁺ pump inhibitor, a ligand for the ouabain/digoxin binding site of the pump, that might mediate the vascular response. In 1991, our group purified EO from human plasma; the substance was identified as ouabain by mass spectrometry. It is now possible to quantify EO by liquid chromatography-tandem mass spectroscopy starting from small (1-mL) samples of human or animal plasma. The liquid chromatography-tandem mass spectroscopy spectra from human and rodent plasma extracts exhibit a major product ion at a mass/charge ratio of 445 (Figure S1 to S3, available in the online data supplement at http://hyper.ahajournals.org); this is the lithiated aglycone of EO (ie, lithiated ouabagenin). The possibility that EO might be the 11β isomer of ouabain is excluded, because the 11-epimers of ouabain have different chromatographic behavior.

Rat adrenal cortex is highly enriched with EO, and human and cow adrenals also contain very high levels. Bilateral adrenalectomy causes a large decline in rat plasma EO, whereas treatment of uninephrectomized rats with deoxycorticosterone acetate (DOCA)+NaCl increases plasma EO >10-fold, and

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Correspondence to Mordecai P. Blaustein, Department of Physiology, University of Maryland School of Medicine, 655 W Baltimore St, Baltimore, MD 21201. E-mail mblaustein@som.umaryland.edu

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significantly elevates BP. These data imply that EO is primarily an adrenocortical hormone, although it may also be synthesized in, and secreted by, the hypothalamus.

Studies of humans and intact animals, and of adrenocortical cell cultures, reveal that EO is synthesized in the adrenal cortex and that its synthesis and secretion are stimulated by adrenocorticotropic hormone (corticotropin [ACTH]). In humans and animals, ACTH-induced hypertension is associated with elevation of EO. Indeed, a preliminary report indicates that certain rare adrenocortical tumors, which are associated with severe hypertension, may produce prodigious amounts of EO.

Approximately 50% of humans with untreated essential hypertension and a majority of patients with adrenocortical adenomas and hypertension have significantly elevated plasma EO; moreover, BP correlates directly with plasma EO. Even in normal human subjects, a high-NaCl diet elevates plasma EO, and a 10-minute infusion of low-dose ouabain increases vascular resistance and elevates BP for >60 minutes.

Plasma EO levels are elevated in several rodent NaCl-sensitive hypertension models, and chronic administration of low-dose ouabain to normal rodents usually induces hypertension in 1 to 3 weeks. Furthermore, subpressor doses of ouabain and DOCA act synergistically to induce hypertension. Ouabain-induced BP elevation in rodents is counteracted by the ouabain antagonist PST-2238 (rostafuroxin). Also, in ACTH, DOCA + NaCl, or reduced renal mass hypertension, Digidig (digoxin-selective Fab fragments that bind ouabain with high affinity) lowers BP.

Interestingly, low-dose ouabain increases TPR in dogs but does not raise BP, presumably because heart rate and CO are markedly reduced. Ouabain also does not induce hypertension in sheep or in mineralocorticoid-resistant Long-Evans rats. These apparent exceptions may, however, yield novel information to help clarify the relationship between EO and hypertension.

Many of the findings cited above provide strong evidence that circulating EO has a key role in the pathogenesis of NaCl-sensitive hypertension. Other studies suggest, however, that brain, not plasma, EO, or even marinobufagenin, may be important.

Surprisingly, digoxin, another cardiotonic steroid and Na,K-ATPase inhibitor, does not induce hypertension in rodents. Also, Digitalis glycosides do not elevate BP in patients treated for congestive heart failure or cardiac arrhythmias. Remarkably, digoxin actually lowers BP in ouabain-hypertensive rats and in many patients with essential hypertension.

Thus, Strophanthus glycosides, such as ouabain, may interact differently with Na+ pumps than do the structurally distinct Digitalis glycosides. Moreover, many observations now indicate that EO is a growth hormone and that it may participate in a variety of kinase-mediated and other signaling pathways independent of its effects on Na+ pump–mediated Na+ transport. EO may, therefore, contribute to vascular remodeling and target organ damage in hypertension. Clearly, there is much that we do not yet understand about the physiology and pharmacology of these agents.

Membrane Microdomains: A Structural Basis for the Action of Ouabain

Na+ pumps are αβ heterodimers. The catalytic subunit, α, contains the Na+, K+, MgATP, and ouabain binding sites and is phosphorylated during each pump cycle. β is essential for pump function; it stabilizes the α subunit conformation and chaperones the αβ complex to the PM. In some tissues, a third subunit, γ, may help to regulate Na+ pump activity. There are 4 mammalian α subunit isoforms (α1 to α4); they are products of different genes but have >90% sequence identity, different expression patterns, and different kinetics, and they are differently regulated. All of the cells express Na+ pumps with an α1 subunit and Na+ pumps with another α isoform. Skeletal, cardiac, and SMs, eg, express Na+ pumps with an α2 subunit, as well as pumps with an α1; most neurons express α1 and α3. Renal epithelia express predominantly (>90% to 95%) Na+ pumps with α1, which mediate the final step in net transepithelial Na+ reabsorption.

The functions of the different α subunit isoforms were elucidated by the discovery that, in a variety of cell types, Na+ pumps with an α2 or α3 subunit are confined to PM microdomains situated adjacent to “junctional” sarcoplasmic/endoplasmic reticulum (js/ER; Figure 1). Here, Na+ pumps colocalize with NCX, which are confined to the same PM microdomains. Na+ pumps with an α1 subunit are more widely distributed in the PM but are apparently excluded from these microdomains. Importantly, the PM microdomains are separated by only 12 to 20 nm from the js/ER, and these structures form a functional unit, termed the “PLasmERosome.” The volume of cytosol in the junctional space between the PM and js/ER of a single PLasmERosome is only on the order of 10−19 to 10−18 L, and diffusion of Na+ and Ca2+ between this space and bulk cytosol is restricted. Thus, standing Na+ and Ca2+ concentration gradients between these compartments and bulk cytosol can be maintained.

Differences in Na+ pump α subunit isoform kinetics play a critical role in PLasmERosome function. The rodent α1 isoform has unusually low affinity for ouabain (Kouabain >100 µmol/L versus <0.05 µmol/L in humans) so that nanomolar ouabain inhibits only the α2 Na+ pumps in rodent arterial myocyte PLasmERosomes. Even in humans, however, where α1 Na+ pumps have high affinity for ouabain, partial inhibition of Na+ pumps by nanomolar ouabain will raise [Na+]i in the junctional space much more than in bulk cytosol. The reason is that the affinity of α2 Na+ pumps for Na+ is much lower (KNa ≈ 22 mmol/L) than the affinity of α1 Na+ pumps (KNa ≈ 12 mmol/L).

The widespread distribution of α1 Na+ pumps implies that they have a “housekeeping” function: they control, primarily, [Na+]i in bulk cytosol. In contrast, the pumps with an α2 (eg, in SM) or α3 catalytic subunit regulate local [Na+]i in the junctional space. Thus, these α2/α3 Na+ pumps control the local Na+ electrochemical gradient that influences Ca2+ transport by NCX. This organizational arrangement (Figure 1) uniquely links cell Ca2+ to Na+ metabolism. The transporters in the PLasmERsome regulate not only [Ca2+]i in the junctional space but S/ER Ca2+ stores and global Ca2+
signaling in the cells as well. Modulation of Na+/K+ pumps in arterial myocyte PLasmERosomes by EO can, therefore, influence arterial tone and BP. Below, we have summarized recent studies in which genetic engineering and pharmacological manipulation of mouse Na+/K+ pumps and NCX (Figure 2) have been used to examine the roles of these transporters in the long-term control of BP.

**Downstream Effector Mechanisms**

**α2 Na⁺ Pumps**

As already noted, chronic administration of exogenous ouabain induces hypertension in rodents. The questions we now address are: how does ouabain (or EO) elevate BP, and is it because of inhibition of SM α2 Na⁺ pumps, as implied above?

**Steps in the Pathogenesis of Salt-dependent Hypertension**

**Etiology:**

- ↑ NaCl Intake
- or Renal Na⁺ Excretion
- or Na⁺ Re-absorption

**Interventions:**

- KIDNEYS
  - ACTH
  - Exogenous Ouabain
    - Ouabain Antagonists
      - “Digibind”
    - Na⁺ Pump α2R
  - Na⁺ Pump α2R, α2smDN
  - Na⁺ Pump α2SMTβ
  - NCX Inhibitors
    - (e.g., SEA0400)
  - NCX1SM/Tg

- ADRENAL CORTEX
  - Na/Ca Exchange (↑ Ca²⁺ Entry)
  - [Ca²⁺]ᵣᵣ

- ARTERIAL SMOOTH MUSCLE
  - Vascular Tone and Contractility
  - ↑ Blood Pressure

**KIDNEYS**

- Plasma Volume

**ADRENAL CORTEX**

- Plasma Endogenous Ouabain (EO)

**ARTERIAL SMOOTH MUSCLE**

- Na/Ca Exchange (↑ Ca²⁺ Entry)

**Green: elevate BP**

**Red: block BP elevation or lower BP**

**Figure 1.** Model of the PM-JS/ER region, the PLasmERosome, showing the location of key transport proteins involved in local control of JS/ER Ca²⁺ stores and Ca²⁺ signaling. The PLasmERosome consists of a PM microdomain, the adjacent JS/ER, and the intervening “diffusion-restricted” junctional space (J). The PM microdomain contains α2 (in SM) or α3 Na⁺ pumps, NCX, and receptor and store-operated channels (ROCs and SOCs, composed of various transient receptor potential channel proteins [TRPCs]). The JS/ER membrane contains SR Ca²⁺ pumps (SERCA), inositol trisphosphate receptors (IP₃Rs), and ryanodine receptors (RYRs). Other regions of the PM contain α1 Na⁺ pumps and Ca²⁺ pumps (PMCas), as well as agonist receptors (ARs; which are G protein–coupled receptors, [GPCRs]). Activation of GPCRs and release of G proteins (GPs) stimulate phospholipase C (PLC) to produce IP₃ and diacylglycerol (DAG). DAG may activate ROCs directly. Na⁺ may enter locally, through ROCs, SOCs, or stretch-activated channels (not shown) to promote Ca²⁺ entry via NCX. Shading indicates relative Na⁺ and/or Ca²⁺ concentrations. (Reprinted with permission).

**Figure 2.** Proposed sequence of steps in the pathogenesis of NaCl-dependent hypertension. The “interventions,” listed at the left, indicate some of the pharmacological and genetic manipulations that were used to test the proposed sequence, as discussed in this review. Genotype nomenclature for genetically engineered mice is given in the text and in legends for Figures 3 and 4. Interventions shown in green increase traffic through the pathway and promote BP elevation; those shown in red block traffic through the pathway and prevent BP elevation or lower BP. Modified from Reference 7.
We found that expression of a short N-terminal segment of the NCX1 (NCX1 SM) knockdown of NCX1 (NCX1 SM) when pressurized to 70 mm Hg.7 Nanomolar ouabain increases in intraluminal pressure and significantly elevated augmented myogenic reactivity in response to stepwise in-pump protein, respectively, in ASM.7 Isolated mesenteric small arteries from the 4 weeks).6 SM-specific overexpression (NCX1SM/Tg)6 had significantly reduced BP. Moreover, the S-specific NCX1 overexpression (NCX1SM/Tg)6 had significantly reduced BP. Mice with a null mutation in one α2 Na+ pump allele (α2SNM/Tg; Song, Chen, Kotlikoff, and Blaustein, unpublished data, 2008) and elevated BP (Figure 3).7 Additionally, the relative BPs of mice with genetically engineered α2 pumps and NCX1. The data, from several sources, are normalized to the BPs of the respective control WT mice. Mice with a null mutation in one α2 Na+ pump allele (α2SM-N/α2WT); Chen, Raina, and Blaustein, unpublished data, 2008) and NCX1SM/Tg mice (8% NaCl–1% NaCl in tap water for 4 weeks).6 SM-specific overexpression of α2 Na+ pumps (α2SM/Tg)9 or Cre-recombinase knockdown of NCX1 (NCX1SM/–) significantly reduced BP. Figure 3. Relative BPs of mice with genetically engineered α2 Na+ pumps and NCX1. The data, from several sources, are normalized to the BPs of the respective control WT mice. Mice with a null mutation in one α2 Na+ pump allele (α2SM-N/α2WT); Chen, Raina, and Blaustein, unpublished data, 2008) and NCX1SM/Tg mice (8% NaCl–1% NaCl in tap water for 4 weeks).6 SM-specific overexpression of α2 Na+ pumps (α2SM/Tg)9 or Cre-recombinase knockdown of NCX1 (NCX1SM/–) significantly reduced BP. P<0.05, **P<0.01 vs WT or the respective genotypes on a normal (0.5%) NaCl diet.

If circulating ouabain (or EO) elevates BP by inhibiting arterial myocyte α2 Na+ pumps, reduced expression of α2 Na+ pumps should have a similar effect. Therefore, we studied mice with a null mutation in either the α1 or α2 Na+ pump.6 Heterozygous (α1+/- and α2+/- or α2S/–; see Figure 2), but not homozygous, null mutants survive, and they express ~50% of the normal complement of α1 or α2 pump protein, respectively, in ASM.7 Isolated mesenteric small arteries from the α2+/- but not α1+/- mice exhibit augmented myogenic response in response to stepwise increases in intraluminal pressure and significantly elevated MT when pressurized to 70 mm Hg.7 Nanomolar ouabain also augments myogenic activity and increases MT with an EC50 of ~1.3 mmol/L.7 Consistent with these effects in isolated arteries, α2+/- but not α1+/- mice have significantly elevated BP (Figure 3).7 Moreover, the α2+/- mice are “NaCl sensitive”: a high-NaCl diet increases BP much more in these mice than in their wild-type (WT) littermates (Figure 3).

The α2+/- mice are “global” single-allele null mutants, but it is important to determine whether the effects are the result of reduced α2 Na+ pump activity/expression in ASM. Recently, we found that expression of a short N-terminal segment of the α2 Na+ pump is dominant negative for expression of full-length α2 pumps.57 Therefore, we generated dominant-negative mice (α2SMDN) that express the α2 N-terminal segment with an SM-specific myosin heavy chain promoter.58 These mice exhibit greatly reduced α2 Na+ pump expression in artery SM (Figure S4, available in the online data supplement at http://hyper.ahajournals.org) and elevated BP (Figure 3).

In a complimentary approach, α1 and α2 Na+ pumps were overexpressed, independently, in mice, under the control of an α-actin SM-specific promoter.59 The mice that overexpressed α2, but not those that overexpressed α1, Na+ pumps had, on average, significantly reduced BP compared with WT mice (Figure 3).

The roles of ouabain/EO and α2 Na+ pumps in elevating BP were also examined in 2 other ways. One type of study used rostafuroxin, a derivative of digitoxigenin,60 that antagonizes the inhibitory action of ouabain on Na,K-ATPase.29 In isolated arteries, rostafuroxin counteracted the augmentation of MT by nanomolar ouabain but not the (ouabain-independent) augmenting effect of reduced α2 expression on MT.7 Rostafuroxin also lowered BP in ouabain-induced hypertension29 and in ≈30% of humans with essential hypertension.29 As an alternative, in a knockin study, 2 amino acids in the α2 Na+ pump ouabain-binding site were mutated to reduce, markedly, the affinity of the α2 pump for ouabain,18,48 Mice that expressed ouabain-resistant α2 pumps (α2K/R) were resistant to ACTH-induced hypertension (Figure 4),18 as well as to ouabain-induced hypertension.48 Moreover, Digibind prevented the ouabain-induced elevation of BP in the WT mice (Figure 4).48 Clearly, ACTH-induced hypertension depends on the existence of a high-affinity cardiacotonic steroid binding site on the α2 Na+ pump and on a water-soluble ligand that binds to this site. The plasma level of this ligand (presumably EO) was increased by ACTH and, like ouabain,32 bound to Digibind with high affinity.48 Moreover, Digibind prevented the ACTH-induced elevation of BP in WT mice. *P<0.01 for the pairings indicated. These regraphed BP data were obtained by tail cuff,14 but comparable results were obtained recently by telemetry (Dostanic-Larson, Lorenz, and Lingrel, unpublished data, 2008).
NCX Type 1

Na/Ca exchange uniquely and directly links Na\(^+\) to Ca\(^{2+}\) metabolism and is a distal regulator of cytosolic Ca\(^{2+}\). There are 2 classes of NCXs, those that cotransport K\(^+\) with Ca\(^{2+}\) and those that do not.\(^6\) The predominant exchanger in arterial myocytes is K\(^-\)-independent NCX, although NCX that cotransports K\(^+\) with Ca\(^{2+}\) has also been detected.\(^6\) There are 3 mammalian NCX isoforms (NCX1 to NCX3), each the product of a different gene.\(^6\) NCX1, which is expressed in ASM, has multiple splice variants; NCX1.3 is the main product of a different gene.\(^6\) NCX1, which is expressed in 3 mammalian NCX isoforms (NCX1 to NCX3), each the product of a different gene.\(^6\) NCX Type 1

Inhibition of Na\(^+\) pumps by nanomolar ouabain augments Ca\(^{2+}\) signaling without elevating bulk cytosolic Na\(^+\) in primary cultured rat arterial myocytes.\(^5\) Even knockout of \(\alpha_2\) Na\(^+\) pumps in cultured cells (astrocytes) has only a minimal effect on bulk cytosolic Na\(^+\) but a large effect on Ca\(^{2+}\) signaling.\(^6\) These findings are consistent with a functional linkage between \(\alpha_2\) (but not \(\alpha_1\)) Na\(^+\) pumps and NCX1, and local reduction of the trans-PM Na\(^+\) gradient when \(\alpha_2\) activity is reduced, as implied by the PLaSMERosome model (Figure 1). Moreover, recent pharmacological and genetic engineering studies now reveal that NCX1 influences not only arterial myocyte Ca\(^{2+}\) metabolism but also long-term vascular tone and BP as well.

Mice in which NCX1 is overexpressed in SM with an \(\alpha\)-actin promoter (NCX1\(^{SM/T8}\)) have elevated BP that is markedly increased by a high-NaCl diet; ie, the mice are NaCl sensitive (Figure 3).\(^6\) The elevated BP in the NCX1 overexpressors on high dietary NaCl is abolished by SEA0400, a selective NCX1 inhibitor,\(^6\) but not if the overexpressed NCX1 contains a G833C mutation,\(^6\) which specifically abrogates the action of SEA0400.\(^6\)

To perform the converse experiment, mice with floxed NCX1 (NCX1\(^{flo/flo}\))\(^6\) were crossed with mice containing a Cre recombinase gene under the control of the SM myosin heavy chain promoter\(^6\) to generate SM-specific NCX1 knockout (NCX1\(^{SM^{-/-}}\)) mice. NCX1\(^{SM^{-/-}}\) mice have abnormally low BP (Figure 3), and isolated, pressurized small arteries from these mice have abnormally low MT.\(^7\) Indeed, SEA0400 also lowers BP by \(~5\) to \(~10\) mm Hg in WT mice\(^6\) and reduces MT by \(~10\)% in isolated arteries from these mice.\(^7\) Thus, NCX1 activity apparently makes a modest but direct contribution to normal MT and BP. SEA0400 also attenuates the increased MT in arteries from \(\alpha_2\)\(^{-/-}\) mice,\(^7\) indicating that NCX1 mediates effects distal to \(\alpha_2\) Na\(^+\) pumps. The BP and MT data from \(\alpha_2\)\(^{-/-}\) and NCX1\(^{SM^{-/-}}\) mice support the view that MT in isolated arteries is an in vitro reflection of BP and, most likely, TPR.

The mice with genetically engineered NCX1 demonstrate that this exchanger contributes to long-term BP regulation: increased NCX1 expression increases BP, whereas knockout of NCX1 reduces BP (Figures 2 and 3). This conclusion is supported by the effects of NCX blockers in several rodent models of NaCl-dependent or ACTH-induced hypertension. In DOCA+NaCl hypertensive rats, spontaneously hypertensive rats on a high-NaCl diet, and Dahl NaCl-sensitive rats on high NaCl, SEA0400 markedly reduces BP.\(^6\) Also, KB-R7943, a less potent NCX blocker, prevents ACTH from elevating BP in mice.\(^1\) Moreover, although a null mutation in one NCX1 allele has a negligible effect on BP (NCX1\(^{+/+}\)) in Figure 3) or MT,\(^7\) it prevents the induction of hypertension by DOCA+NaCl.\(^6\) Importantly, SEA0400 did not lower BP in several NaCl-independent rat hypertension models: spontaneously hypertensive rats on a normal NaCl diet, stroke-prone spontaneously hypertensive rats, and the renin-dependent 2-kidney/1-clip rat.\(^6\) The implication is that NCX1 makes an important contribution to the pathogenesis of NaCl-dependent hypertension but not to NaCl-independent hypertension.

“Kalzium? Ja, Das ist Alles!” (Calcium Is Everything: O. Loewi)

Arterial myocyte contraction depends, ultimately, on the availability of cytosolic Ca\(^{2+}\);\(^8\) and the sensitivity of the contractile apparatus to that Ca\(^{2+}\);\(^7\) Furthermore, NCX1, under the control of the Na\(^+\) gradient generated by the adjacent \(\alpha_2\) Na\(^+\) pumps, helps regulate myocyte Ca\(^{2+}\) ho-
meostasis (Figure 1). For example, the nanomolar ouabain-induced increase in MT is associated with increased myocyte [Ca\(^{2+}\)]; conversely, reduction of MT by SEAO4000 is associated with reduced myocyte [Ca\(^{2+}\)] (Figure 5). Thus, it is apparent that α2 Na\(^{+}\) pumps and NCX1 are relatively distal mechanisms in the final common path that links NaCl to vasoconstriction and hypertension (Figure 2). Indeed, all upstream vasoconstrictr and vasodilator mechanisms (neural and humoral) must, inevitably, be influenced by the activity of these 2 transporters, because they regulate basal [Ca\(^{2+}\)]\(_{\text{CYT}}\) in arterial myocytes.

An alternative suggestion is that activation of Rho/Rho kinase via the G\(_{12}\)-G\(_{13}\)-mediated G protein–coupled receptor pathway, which modulates the Ca\(^{2+}\) sensitivity of the contractile apparatus in ASM,\(^7\) is selective for NaCl-dependent hypertension.\(^7\) Those authors, however, studied only an NaCl-dependent (DOCA+NaCl) mouse model; they did not test whether the G\(_{12}\)-G\(_{13}\) pathway also operates in NaCl-independent forms of hypertension.\(^7\) In fact, interference with the G\(_{12}\)-G\(_{13}\) pathway, whether at the agonist receptor level\(^7\) or at the level of the Rho kinase,\(^7\) lowers BP in NaCl-independent models, such as the stroke-prone spontaneously hypertensive rat\(^7\) and the NO synthase-inhibited rat.\(^7\) The G\(_{12}\)-G\(_{13}\) pathway is, therefore, downstream and distinct from the key NaCl-sensitive steps in Na\(^{+}\)-dependent hypertension. Once NaCl-sensitive, NCX1-mediated Ca\(^{2+}\) entry has occurred, the G\(_{12}\)-G\(_{13}\) pathway helps modulate the increases in vascular tone and BP.

**Endgame: Na/Ca Exchange, Ca\(^{2+}\) Entry, and Myogenic Tone**

In the heart, the main role of NCX is to extrude, during diastole, much of the Ca\(^{2+}\) that enters through voltage-gated channels during systole.\(^7\) Consequently, reduced cardiac NCX1 function as a result, eg, of α2 Na\(^{+}\) pump inhibition by cardiotoxic steroids, is associated with Ca\(^{2+}\) gain and augmented signaling in cardiac myocytes. Therefore, it might at first seem surprising that ASM NCX1 contributes directly to vascular tone and that reduced expression or pharmacological inhibition of NCX1 in arterial myocytes lowers [Ca\(^{2+}\)]\(_{\text{CYT}}\) and attenuates Ca\(^{2+}\) signaling (Figure 5). Indeed, Ca\(^{2+}\) entry via NCX has sometimes been called “reverse mode” exchange, implying, erroneously, that this is the backward or abnormal operation of the exchanger.\(^7\) NCX can transport Ca\(^{2+}\) in either direction across the PM,\(^7\) under the control of the local Na\(^{+}\) electrochemical gradient across the PM (Figure 1), and considerations of the electric component of this gradient are of paramount importance. In the heart, the driving force on the exchanger, ie, the difference between the prevailing membrane voltage (V\(_{\text{M}}\)) and the NCX1 reversal potential (E\(_{\text{Na/Ca}}\)), which determines the direction of net Ca\(^{2+}\) movement, varies during the cardiac cycle. (For NCX1, which mediates the exchange of 3Na\(^{+}\) for 1Ca\(^{2+}\), E\(_{\text{Na/Ca}}\)=\(3E_{\text{Na}}-2E_{\text{Ca}}\), where E\(_{\text{Na}}\) and E\(_{\text{Ca}}\) are, respectively, the equilibrium potentials for Na\(^{+}\) and Ca\(^{2+}\), E\(_{\text{Na}}\)=(RT/F) ln (Na\(^{+}\)/Na\(^{-}\)) and E\(_{\text{Ca}}\)=(RT/2F) ln (Ca\(^{2+}\)/Ca\(^{-}\)), and R, T, and F are the gas constant, temperature in degrees Kelvin, and Faraday’s number, respectively.\(^7\) The rapid membrane depolarization during the upstroke of the cardiac action potential, for example, rapidly switches NCX1 from the Ca\(^{2+}\) exit to Ca\(^{2+}\) entry mode, as the driving force, V\(_{\text{M}}\)-E\(_{\text{Na/Ca}}\), becomes positive. Then, as V\(_{\text{M}}\) repolarizes, during diastole, V\(_{\text{M}}\)-E\(_{\text{Na/Ca}}\) again becomes negative and favors Ca\(^{2+}\) exit.\(^7\)

A different situation exists in ASM, where changes in V\(_{\text{M}}\) are normally quite slow, and cells are often partially depolarized for very long periods of time.\(^7\) Here, intraluminal pressure in small arteries depolarizes the myocytes and activates dihydropyridine-sensitive L-type voltage-gated channels. Opening of stretch-activated nonselective cation channels\(^8\) may initiate the depolarization. This depolarization is insensitive to dihydropyridines: nifedipine blocks Ca\(^{2+}\) entry through L-type voltage-gated channels and reduces MT but has little effect on the pressure-activated depolarization.\(^7\) The Na\(^{+}\) entry through stretch-activated channels and consequent depolarization, as well as the rise in [Ca\(^{2+}\)]\(_{\text{CYT}}\), should also have another, previously unrecognized consequence: they should promote Ca\(^{2+}\) entry via NCX1 and thereby contribute to MT. The reason is that the exchanger is activated by cytosolic Ca\(^{2+}\),\(^8\) and the rise in cytosolic [Na\(^{+}\)] and the depolarization augment the Ca\(^{2+}\) entry mode of NCX1 by increasing the driving force, V\(_{\text{M}}\)-E\(_{\text{Na/Ca}}\). The implication is that both the L-type voltage-gated channels and NCX1 contribute to the maintenance of Ca\(^{2+}\) entry, elevated [Ca\(^{2+}\)]\(_{\text{CYT}}\), and arterial tone when the arteries are pressurized.

“In My End Is My Beginning” (T.S. Eliot)

In this review, we have explored some of the critical steps that link NaCl retention with the long-term increase in TPR and elevation of BP. Recent results, especially those from chemical analyses of human and rodent plasma samples and from genetic engineering and pharmacological studies in rodents and rodent arteries are summarized above. These studies give new insight into some of the molecular events that help regulate cytosolic Ca\(^{2+}\) and vascular tone. The data supply compelling evidence that EO, SM α2 Na\(^{+}\) pumps, and NCX1 are key mechanisms in the pathway that leads from NaCl retention to hypertension (Figure 2).

These findings provide a framework, but the story is far from complete. For example, a key area where knowledge is lacking is at the early steps between NaCl retention and the release of EO, as indicated by the broken vertical lines in Figure 2. Also, Crowley et al\(^8\) demonstrated recently that the renal and extrarenal arteries make apparently independent (and equal) contributions to the long-term regulation of BP, but how the distal mechanisms, discussed above, affect the renal and extrarenal vasculature and renal function and thereby contribute to BP control is still unexplored. Finally, of course, a fundamental question is: what makes us NaCl sensitive in the first place? Hopefully, the progress outlined above will clarify new directions for hypertension research to help resolve these issues.

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None.

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The Pump, the Exchanger, and Endogenous Ouabain: Signaling Mechanisms That Link Salt Retention to Hypertension

Mordecai P. Blaustein, Jin Zhang, Ling Chen, Hong Song, Hema Raina, Stephen P. Kinsey, Michelle Izuka, Takahiro Iwamoto, Michael I. Kotlikoff, Jerry B. Lingrel, Kenneth D. Philipson, W. Gil Wier and John M. Hamlyn

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The Pump, the Exchanger and Endogenous Ouabain: Signaling Mechanisms that Link Salt Retention to Hypertension

Mordecai P. Blaustein¹,², Jin Zhang¹, Ling Chen², Hong Song¹, Hema Raina¹, Stephen P. Kinsey¹, Michelle Izuka¹, Takahiro Iwamoto³, Michael I. Kotlikoff⁴, Jerry B. Lingrel⁵, Kenneth D. Philipson⁶, W. Gil Wier¹ and John M. Hamlyn¹

Departments of ¹Physiology and ²Medicine, and the ¹Center for Heart, Hypertension & Kidney Disease, University of Maryland School of Medicine, Baltimore, MD, USA;
³Department of Pharmacology, Fukuoka University School of Medicine, Fukuoka, Japan;
⁴Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA;
⁵Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH, USA;
⁶Department of Physiology and the Cardiovascular Research Laboratories, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA
Assay of Endogenous and Exogenous Ouabain.

Mass spectroscopy (MS) methods reveal that the endogenous ouabain (EO) isolated from human plasma has a mass of 584.2 daltons, identical to that obtained for plant-derived ouabain.\(^1\) Advances in MS instrumentation, coupled with improved understanding of the behavior of various ion adducts of EO in the gas phase, now enable quantitation and fingerprinting of EO using small clinically relevant volumes of blood.\(^2\) Examples of the liquid chromatography-tandem MS-MS (LC-MS-MS) of endogenous ouabain from 0.25 ml rat plasma and plant derived ouabain are shown in Supplementary Figures 1 and 2, respectively.

Inspection of the key ion current chromatograms and the MS-MS spectra prove the presence of EO in normal rat plasma and show (in this instance) that it circulates at the high end of the subnanomolar range as documented by prior RIA and bioassay methods.\(^3\)

References


Supplementary Figure 1. Detection and Quantitation of Endogenous Ouabain in Rat Plasma by LC-MS-MS. A 10 ml of fresh rat plasma was extracted over C18 as described. Following reconstitution, an aliquot corresponding to only 0.25 ml of the original plasma was injected into a capillary C-18 column attached to a liquid chromatograph (Agilent 1100) interfaced with a Bruker Esquire Ion Trap Mass Spectrometer. A solvent gradient program was used to elute the bound materials which in turn were continuously monitored for positive ion species over an abbreviated scan range (400-650 m/z). In addition, selective ion monitoring was performed for positive ions equivalent to lithiated ouabain (i.e., m/z = 591.3). The top panel (red) shows the summed MS ion chromatogram for positive ions within the scanned range (i.e., 400-650 m/z). The second panel (green) shows the extracted MS ion current chromatogram for positively charged molecular ions with m/z = 591.3 (i.e., equivalent to lithiated EO). The third panel (blue) shows the extracted MS-MS ion current chromatogram resulting from the collision induced dissociation (CID) of all ions with 591.3 m/z. Note the prominent peak eluting at 27.9 minutes; the MS-MS spectrum of that ion peak is shown in the bottom panel (black). The targeted CID of the EO parent ion at m/z 591.3 led to formation of characteristic product ions at m/z 445.2, 427.2 and 409.2 (arrows) representing the lithiated aglycone of EO
and its two dehydrated derivatives, respectively. Interpolation of the MS-MS ion
current at 27.9 minutes with a standard calibration of the LC-MS-MS using
ouabain under identical conditions (not shown) indicated that the EO content of
the rat plasma sample was 141 pmoles/L. (Hamlyn and Manunta, unpublished).
Supplementary Figure 2. LC-MS-MS of Ouabain. Following analysis of the rat plasma sample in Supplementary Figure 1, ouabain (75 fmoles) was injected into the LC. The elution conditions, mass spectrometer settings, and ion monitoring conditions were identical to those used in Supplementary Figure 1. The top panel (red) shows the summed MS ion chromatogram for positively charged ions within the scanned range (i.e., 400-650 m/z). The second panel (green) shows the extracted MS ion current chromatogram for positively charged molecular ions with m/z = 591.3 (i.e., equivalent to lithiated ouabain). The third panel (blue) shows the extracted MS-MS ion current chromatogram resulting from the collision induced dissociation (CID) of ions with 591.3 m/z (the m/z of lithiated ouabain). A prominent ion current peak eluted at 27.9 minutes and the MS-MS spectrum of that ion peak shown in the bottom panel (black) reveals product ions at m/z 445.2, 427.2 and 409.2 (arrows) equivalent to the lithiated aglycone of ouabain and its singly and doubly dehydrated derivatives, respectively. (Hamlyn and Manunta, unpublished).
Supplementary Figure 3. Endogenous ouabain in human plasma determined by LC-MS-MS. A. LC-MS-MS of human plasma following LC separation and collision-induced dissociation of lithiated molecular ions at m/z 591 ± 0.5; the extracted ion current chromatogram for lithiated product ions was monitored at m/z 445. The prominent product ion current at 53 min corresponds with the elution of the EO aglycone, ouabagenin, following dissociation of EO. B. Mass spectrum of the product ions eluted at 53 min. The diamond at m/z 591 corresponds to the target ion (lithiated ouabain/EO). Product molecular ions at m/z 445.2, and 427.3 correspond to the lithiated aglycone of EO and its monodehydrated derivative, respectively.
Supplementary Figure 4. Immunoblots of aorta and bladder smooth muscle, and brain, membranes from wild type (C) and smooth muscle-specific α2 dominant negative (α2SM/DN) mice (DN). An α2(1-120)Flag construct, the N-terminal 120 amino acid residues of the α2 Na+ pump, under the control of a smooth muscle myosin heavy chain promoter, was expressed in the α2SM/DN mice. The construct expression was detected with anti-Flag antibodies in smooth muscle (bladder; insufficient aorta protein was available), but not in brain. The Na+ pump α2 subunit (detected with anti-α2 HERED antibodies) was down-regulated in both smooth muscles, but not in brain. Lane protein content was controlled with α-actin (Song, Chen, Kotlikoff and Blaustein, unpublished).