Sodium pump $\alpha 2$ subunits control myogenic tone and blood pressure in mice

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A key question in hypertension is: How is long-term blood pressure controlled? A clue is that chronic salt retention elevates an endogenous ouabain-like compound (EOLC) and induces salt-dependent hypertension mediated by Na^+/Ca^{2+} exchange (NCX). The precise mechanism, however, is unresolved. Here we study blood pressure and isolated small arteries of mice with reduced expression of Na^+ pump $\alpha 1$ ($\alpha 1^{+/-}$) or $\alpha 2$ ($\alpha 2^{+/-}$) catalytic subunits. Both low-dose ouabain (1–100 nM; inhibits only $\alpha 2$) and high-dose ouabain (≥ 1 μM; inhibits $\alpha 1$) elevate myocyte Ca^{2+} and constrict arteries from $\alpha 1^{+/-}$, as well as $\alpha 2^{+/-}$ and wild-type mice. Nevertheless, only mice with reduced $\alpha 2$ Na^+ pump activity ($\alpha 2^{+/-}$), and not $\alpha 1$ ($\alpha 1^{+/-}$), have elevated blood pressure. Also, isolated, pressurized arteries from $\alpha 2^{+/-}$, but not $\alpha 1^{+/-}$, have increased myogenic tone. Ouabain antagonists (PST 2238 and canrenone) and NCX blockers (SEA0400 and KB-R7943) normalize myogenic tone in ouabain-treated arteries. Only the NCX blockers normalize the elevated myogenic tone in $\alpha 2^{+/-}$ arteries because this tone is ouabain independent. All four agents are known to lower blood pressure in salt-dependent and ouabain-induced hypertension. Thus, chronically reduced $\alpha 2$ activity ($\alpha 2^{+/-}$ or chronic ouabain) apparently regulates myogenic tone and long-term blood pressure whereas reduced $\alpha 1$ activity ($\alpha 1^{+/-}$) plays no persistent role: the *in vivo* changes in blood pressure reflect the *in vitro* changes in myogenic tone. Accordingly, in salt-dependent hypertension, EOLC probably increases vascular resistance and blood pressure by reducing $\alpha 2$ Na^+ pump activity and promoting Ca^{2+} entry via NCX in myocytes.

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Elevated blood pressure (BP), hypertension, is prevalent in developed societies, and is a major risk factor for disability and death (Kaplan, 2002; Chobanian *et al.* 2003). Salt (NaCl) retention by the kidneys typically leads to hypertension (Guyton, 1990; Kaplan, 2002; Johnson *et al.* 2005). Indeed, monogenic diseases of renal salt retention raise BP; in contrast, salt wasting syndromes lower BP (Lifton *et al.* 2001). Mutation, knockout or duplication of genes that affect BP induce either salt-dependent hypertension or unusual forms of salt-independent hypertension (Takahashi & Smithies, 1999). In essential hypertension, the primary defect may be an acquired renal injury rather than a genetic defect (Johnson *et al.* 2005). Nevertheless, none of those studies have addressed the question of precisely how salt retention leads to chronic hypertension (Kaplan, 2002; Johnson *et al.* 2005). In this paper we elucidate downstream molecular mechanisms and clarify the link between salt and hypertension.

Mean arterial BP depends primarily on cardiac output (CO) and total peripheral systemic vascular resistance (TPR) (Berne & Levy, 2001): at constant CO, mean BP $\approx$ CO $\times$ TPR. Acute plasma volume expansion elevates BP by increasing CO (Borst & Borst-de Geus, 1963; Guyton, 1990). With sustained volume expansion,

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however, TPR rises to maintain the elevated BP while CO declines (Borst & Borst-de Geus, 1963; Guyton, 1990). This condition of high TPR and near-normal CO is commonly observed in humans with essential hypertension (Cowley, 1992; Kaplan, 2002). Nevertheless, long-term control of BP is still poorly understood.

The shift from high CO to high TPR, called 'whole-body autoregulation', has been attributed to regulation of blood flow to meet metabolic demand (Guyton, 1990; Kaplan, 2002). This view is controversial (Julius, 1988), however, and the mechanisms are unresolved (Kaplan, 2002; Johnson *et al.* 2005). According to one hypothesis (Fig. 1) (Blaustein, 1977), salt retention promotes secretion of an endogenous cardiotonic (and vasotonic) steroid that inhibits Na^+ pumps, including those in vascular smooth muscle. By raising the cytosolic Na^+ concentration ($[Na^+]_{cyt}$), this agent would be expected to promote Na^+/Ca^{2+} exchanger (NCX)-mediated Ca^{2+} entry into the myocytes. This should elevate the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$), and thus increase TPR by enhancing myogenic tone, the intraluminal pressure-induced intrinsic arterial constriction that is prominent in small resistance arteries (Hill *et al.* 2001). Indeed, recent evidence reveals that NCX type-1 (NCX1) in arterial myocytes plays a central role in ouabain-induced hypertension and salt-dependent hypertension (Iwamoto *et al.* 2004*b*).

The discovery of an endogenous ouabain-like compound (EOLC) that is synthesized and secreted by the adrenal cortex (Hamlyn *et al.* 1991, 2003; Schoner, 2002) supports the hypothesis presented in Fig. 1. Plasma EOLC levels are elevated in ∼45% of patients with essential hypertension (Rossi *et al.* 1995; Ferrandi *et al.* 1998; Manunta *et al.* 1999; Goto & Yamada, 2000; Pierdomenico *et al.* 2001) and in several animal models of salt-dependent hypertension (Hamlyn *et al.* 1991; Ferrandi *et al.* 1998; Takada *et al.* 1998). The EOLC levels correlate with BP (Rossi *et al.* 1995; Manunta *et al.* 1999; Goto & Yamada, 2000). Moreover, prolonged administration of ouabain, the Na^+ pump inhibitor from plants, induces sustained, dose-dependent increases in TPR and BP in normal rats and mice (Yuan *et al.* 1993; Manunta *et al.* 1994; Schoner, 2002; Iwamoto *et al.* 2004*b*; Dostanic *et al.* 2005).

Na^+ pumps are expressed as $\alpha\beta$ dimers (Blanco & Mercer, 1998). Four isoforms of the catalytic (α) subunit, the only known ouabain receptor, have been identified (Blanco & Mercer, 1998), but mouse arteries only express Na^+ pumps with the $\alpha1$ and $\alpha2$ isoforms (Shelly *et al.* 2004). Rodent $\alpha1$ has unusually low ouabain affinity ($EC_{50} > 50\ \mu$M) (O'Brien *et al.* 1994; Blanco & Mercer, 1998) whereas, in mammals, Na^+ pumps with $\alpha2$ subunits have high ouabain affinity ($EC_{50} < 50$ nM) (O'Brien *et al.* 1994; Blanco & Mercer, 1998).

Here we show that exogenous ouabain, at low concentrations approaching circulating EOLC levels, elevates $[Ca^{2+}]_{cyt}$ and augments vasoconstriction of pressurized small arteries. Moreover, mice heterozygous for $\alpha2$ Na^+ pumps (James *et al.* 1999) ($\alpha2^{+/-}$, which mimic the effects of nanomolar ouabain), but not mice heterozygous for $\alpha1$ ($\alpha1^{+/-}$), have altered artery function

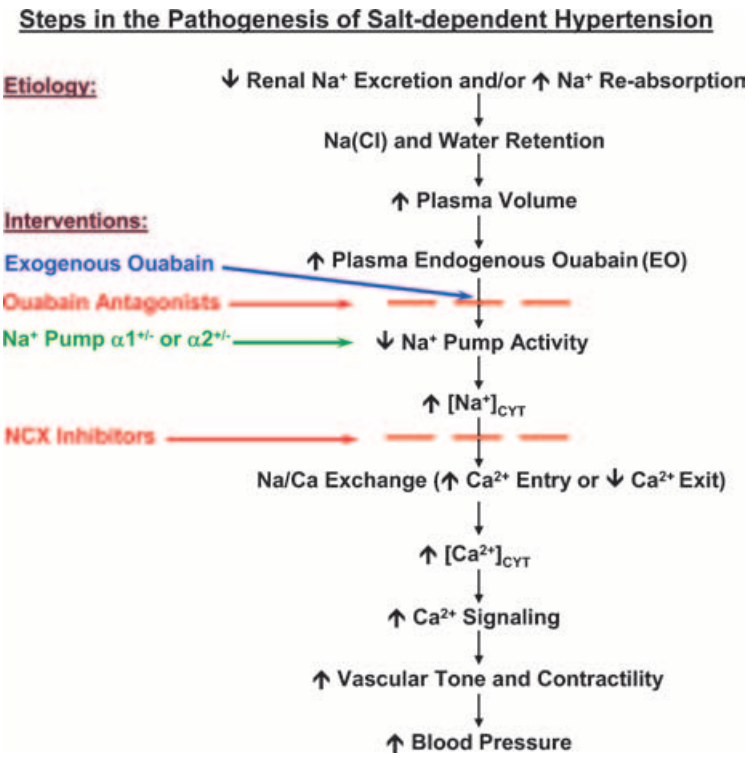

Figure 1. Proposed mechanism for the pathogenesis of salt-dependent hypertension
Interventions such as chronic administration of exogenous ouabain, use of heterozygous null mutant mice and treatment with agents that interfere with ouabain's action or the Na^+/Ca^{2+} exchange (NCX) are indicated on the left.

and elevated BP. These data demonstrate, for the first time, that modulation of $\alpha 2$ Na^+ pump activity, and not $\alpha 1$, regulates small artery contractility and exerts long-term control over BP (Fig. 1).

Methods

Experimental animals

Wild-type (WT) C57/BL6 mice and mice with a null mutation in one Na^+ pump $\alpha 1$ or $\alpha 2$ gene ($\alpha 1^{+/-}$ or $\alpha 2^{+/-}$) were studied; the homozygous knockouts do not survive (James *et al.* 1999). Genomic DNA was obtained from tail biopsies for genotyping by PCR.

In some experiments, normal male Sprague-Dawley rats (150–240 g) were used. All rat and mouse protocols were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

Haemodynamic measurements

Mice (~16 weeks old) were anaesthetized with isoflurane supplemented with 100% O_2; core temperature was maintained at 37.5–38°C. The right femoral artery was surgically isolated and cannulated with a 1.4 F Mikro-tip pressure catheter (Millar Instruments, Houston, TX, USA). Blood pressure was acquired under 1.5% isoflurane anaesthesia (Janssen *et al.* 2004); data were calculated off-line (BioPac System, Santa Barbara, CA, USA). After the experiment the animal was killed by cervical dislocation following an isofluorane overdose. The data collection was performed 'double-blind': the individual who instrumented and measured the BP did not know the genotype. Animal code numbers were matched with genotype and BP after the data on all the mice had been collected.

Diameter and $[Ca^{2+}]_{cyt}$ measurements

Mice and rats were killed by rapid cervical dislocation and decapitation. Mesenteric small arteries were isolated and pressurized to permit myogenic tone development (Zhang *et al.* 2002). Myogenic tone was generated at an intraluminal pressure of 70 mmHg unless otherwise noted. External diameter was monitored with a Nikon (Melville, NY, USA) TMS microscope (×10 objective) and a monochrome CCD camera operated by LabView software (National Instruments, Austin, TX, USA) (Zhang *et al.* 2002). Passive external diameter was measured in Ca^{2+}-free solution (Zhang *et al.* 2002). Myogenic reactivity was determined by measuring the steady-state diameters following step changes in intraluminal pressure.

For $[Ca^{2+}]_{cyt}$ measurement, pressurized arteries were loaded (room temperature) with 10 μM fura-2 at 20 mmHg for 45 min or 10 μM fluo-4 at 70 mmHg for 2 h in albumin-free dissection solution containing 1.0% DMSO (vol/vol) and 0.03% cremaphor EL (vol/vol).

Fluo-4 imaging. Arteries were imaged in one of two scanning planes (Mauban *et al.* 2001; Zhang *et al.* 2002) with a Nipkow-Yokogawa spinning disc confocal imaging system (CSU10, Solomere Technology, Salt Lake City, UT, USA) connected to a Stanford XR-Mega 10 camera (Stanford Photonics, Palo Alto, CA, USA). The spinning disc was mounted on a Nikon Eclipse TE2000-U inverted microscope (×60, numerical aperture (NA) 1.2 water immersion objective). The confocal images shown here were obtained from an optical plane at the centre of the artery, parallel to the long axis. In this plane, the myocyte cross-sections in the artery walls remain in the plane of focus while the artery walls move horizontally during vasoconstriction and vasodilatation (Mauban *et al.* 2001). This permits simultaneous diameter determination and Ca^{2+} imaging (e.g. Fig. 2*A*); the vasoconstriction of dye-loaded arteries, however, is often somewhat attenuated (see Results section).

Fura-2 imaging. Arteries were excited alternately at 340 and 380 nm by a Lambda DG-4 illumination system (Sutter Instruments, Novato, CA, USA) and were viewed with a TE2000-U inverted microscope (×40 oil objective). The myocytes in the artery walls were imaged in a focal plane tangential to the surface of the artery close to the floor of the tissue chamber (Mauban *et al.* 2001; Zhang *et al.* 2002). Images were acquired with an ORCA-ER camera (Hamamatsu Corp., Bridgewater, NJ, USA) using MetaFluor software (Universal Imaging, Chester, PA, USA). Individual myocyte $[Ca^{2+}]_{cyt}$ was calculated using Grynkiewicz's equation:

$$[Ca^{2+}]_{cyt} = K_d \beta (R - R_{min})/(R_{max} - R)$$

with an apparent dissociation constant (K_d) of 282 (Knot & Nelson, 1998) after *in situ* calibration. R_{min}, R_{max} and β were determined for each region where R is the fluorescent emission ratio with excitation at 340 and 380 nm, R_{min} and R_{max} are the emission ratios under Ca^{2+}- free (5mM EGTA and saturating (24 mM) Ca^{2+} conditions respectively, and β is the emission ratio with 380 nm excitation under Ca^{2+}-free and saturating Ca^{2+} conditions.

Membrane potential measurements

Membrane potential was recorded in rat mesenteric small arteries (140–200 μm external diameter) using standard sharp microelectrodes. Pipettes were pulled on a Brown-Flaming electrode puller (Sutter Instruments) and filled with 3 M KCl (resistance, 70–100 MΩ). The preamplifier (M-707A, World Precision Instruments, Sarasota, FL, USA) output was digitized using a Digidata 1322A (Axon Instruments, Union City, CA, USA);

data were analysed using pCLAMP software (Axon Instruments).

Western blotting

Mesenteric arteries, hearts and kidneys were minced and homogenized in NaCl/sucrose buffer and membrane fractions were prepared (Lencesova *et al.* 2004). Tissue extracts were analysed by Western blot with polyclonal α-isoform-specific and non-selective antibodies (Pressley, 1992); the antibodies were gifts from Drs T. Pressley (Texas Tech University, Lubbock, TX, USA) and A. McDonough (University of Southern California, Los Angeles, CA, USA). Band densities were quantified (Golovina *et al.* 2003) using Kodak ID image analysis software (Eastman Kodak, Rochester, NY, USA). For these analyses, we confirmed that the amounts of protein in the bands were within the linear range of signal intensities. Band densities were normalized with β-actin for arterial and renal membranes, and glyceraldehyde-3-phosphate dehydrogenase for cardiac membranes. The relative amounts of $\alpha 1$ and $\alpha 2$ in the tissue samples were calculated on the basis of evidence that 80–87% of the Na^+ pumps in skeletal muscle have an $\alpha 2$ subunit, and the remainder is $\alpha 1$ (He *et al.* 2001; Golovina *et al.* 2003).

Reagents and solutions

Artery dissection solution (mM): NaCl, 145; KCl, 4.7; $MgSO_4 \cdot 7H_2O$, 1.2; Mops, 2.0; EDTA, 0.02; NaH_2PO_4, 1.2; $CaCl_2 \cdot 2H_2O$, 2.0; glucose, 5.0; pyruvate, 2.0; with 1% albumin (pH 7.4 at 5°C). Krebs perfusion solution (mM): NaCl, 112; $NaHCO_3$, 26; KCl, 4.9; $CaCl_2$, 2.5; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; glucose, 11.5; Hepes, 10 (pH adjusted to 7.3–7.4 with NaOH). Ca^{2+}-free solution was made by omitting Ca^{2+} and adding 0.5 mM EGTA. Solutions were gassed with 5% O_2, 5% CO_2 and 90% N_2. Solution for membrane potential measurement (mM): NaCl, 140; KCl, 5; NaH_2PO_4, 1.2; $MgCl_2$, 1.4; Hepes, 10; $NaHCO_3$, 5; $CaCl_2$, 1.8; glucose, 11.5 (pH adjusted to 7.3 with NaOH).

Reagents and sources were as follows: ouabain, phenylepherine, phentolamine, acetylcholine and cremaphor EL (Sigma-Aldrich, St Louis, MO, USA); SEA0400 (Taisho, Tokyo, Japan); PST 2238 (Prassis/Sigma Tau, Milan, Italy); canrenone (Pharmacia Ltd, Morpeth, Northumberland, UK); KB-R7943 (Tocris, Ellisville, MO, USA); fluo-4 and fura-2 (Molecular Probes, Eugene, OR, USA). Other reagents were reagent grade or the highest grade available.

Data analysis and statistics

The data are expressed as means ± S.E.; n denotes the number of arteries studied (one per animal) unless otherwise stated. Comparisons of data were made using Student's paired or unpaired t test, as appropriate; one-way or two-way ANOVA was used where indicated (see figure legends). Differences were considered significant at $P < 0.05$. Images were analysed with customized Interactive Data Language software (IDL, Research Systems, Inc., Boulder, CO, USA).

Results

Nanomolar ouabain augments myogenic tone

WT mouse mesenteric small arteries were loaded with the Ca^{2+} indicators, fluo-4 (Fig. 2*A*, *C* and *D*) or fura-2 (Fig. 2*E*). Fluo-4 fluorescence, a measure of $[Ca^{2+}]_{cyt}$, was relatively low in relaxed myocytes at 23°C (Fig. 2*A*). Cross-sections of individual myocytes are seen (arrows) in these confocal optical cross-sections of the artery wall (Zhang *et al.* 2002) that contains only a single layer of myocytes.

$[Ca^{2+}]_{cyt}$ rose (i.e. fluorescence increased) after a 40–50 min delay when the arteries were pressurized to 70 mmHg and temperature was increased from 23 to 35°C. This was followed (Fig. 2*Ab*) by vasoconstriction (i.e. myogenic tone) (Hill *et al.* 2001). Under these conditions, but in the absence of Ca^{2+} indicator, the arteries constricted from 129 ± 1 μm (passive external diameter) to 99 ± 2 μm ($n = 91$); thus, myogenic tone at 70 mmHg in WT arteries was a 23 ± 1% constriction from passive diameter. Vasoconstriction in Ca^{2+} indicator-loaded arteries was usually attenuated, perhaps because of Ca^{2+} buffering by the indicator.

In arteries with myogenic tone, 100 nM ouabain induced a further, reversible constriction (Fig. 2*B*). On average, ouabain decreased external diameter from 101 ± 1 to 93 ± 1 μm; i.e. myogenic tone increased from 23 ± 1% of passive diameter to 29 ± 1% of passive diameter, a 25 ± 2% increase ($n = 64$; $P < 0.001$). This constriction corresponds to a 10 μm decrease in internal diameter, from 85 to 75 μm.

The arteries were exposed to ouabain for periods of only 2–10 min in these and most other experiments. In experiments in which the myogenic response to step increases in pressure was examined (see below), however, the exposure to ouabain was prolonged. In these arteries, myogenic tone at 70 mmHg was 27 ± 3% of passive diameter after 10 min, and 32 ± 3% ($n = 5$) after 50–60 min of treatment with 100 nM ouabain. Thus, this effect of ouabain on myogenic tone was maintained (or even slightly increased), in contrast to the transient response described in noradrenaline-contracted small arteries (Aalkjaer & Mulvany, 1985).

Ouabain did not constrict pressurized arteries before the generation of myogenic tone (Fig. 2*B*). Thus, the ouabain-induced vasoconstriction apparently depended

upon some of the same cellular mechanisms that produce myogenic tone: elevation of $[Ca^{2+}]_{cyt}$ (Fig. 2*A*) and/or enhanced Ca^{2+} sensitivity of the contractile apparatus (Hill *et al.* 2001). Ouabain (100 nM) elevated $[Ca^{2+}]_{cyt}$ in pressurized arteries before (Fig. 2*C*) as well as after (Fig. 2*D*) the generation of myogenic tone but, in the latter case, the ouabain-induced effect was superimposed on a significantly higher prior $[Ca^{2+}]_{cyt}$ (Fig. 2*E*). Even in two pressurized arteries treated with 0.3 μM nifedipine, which markedly reduces $[Ca^{2+}]_{cyt}$ and myogenic tone (Zhang *et al.* 2002), 100 nM ouabain still constricted the arteries by 6 and 20% of the diameter with nifedipine alone. Therefore, both an appropriately high $[Ca^{2+}]_{cyt}$ and a pressure-induced increase in Ca^{2+} sensitivity may contribute to the ouabain-induced constriction.

Fluo-4 is a non-ratiometric Ca^{2+} indicator and does not report absolute $[Ca^{2+}]_{cyt}$. The ratiometric Ca^{2+} sensor, fura-2, was therefore used to determine $[Ca^{2+}]_{cyt}$ in individual myocytes imaged in a plane tangential to the surface of the artery (Fig. 2*E*). $[Ca^{2+}]_{cyt}$ was 185 nM at 70 mmHg. Ouabain (100 nM) reversibly elevated $[Ca^{2+}]_{cyt}$ by 22 ± 4 nM ($n = 22$ cells; $P < 0.001$); thus,

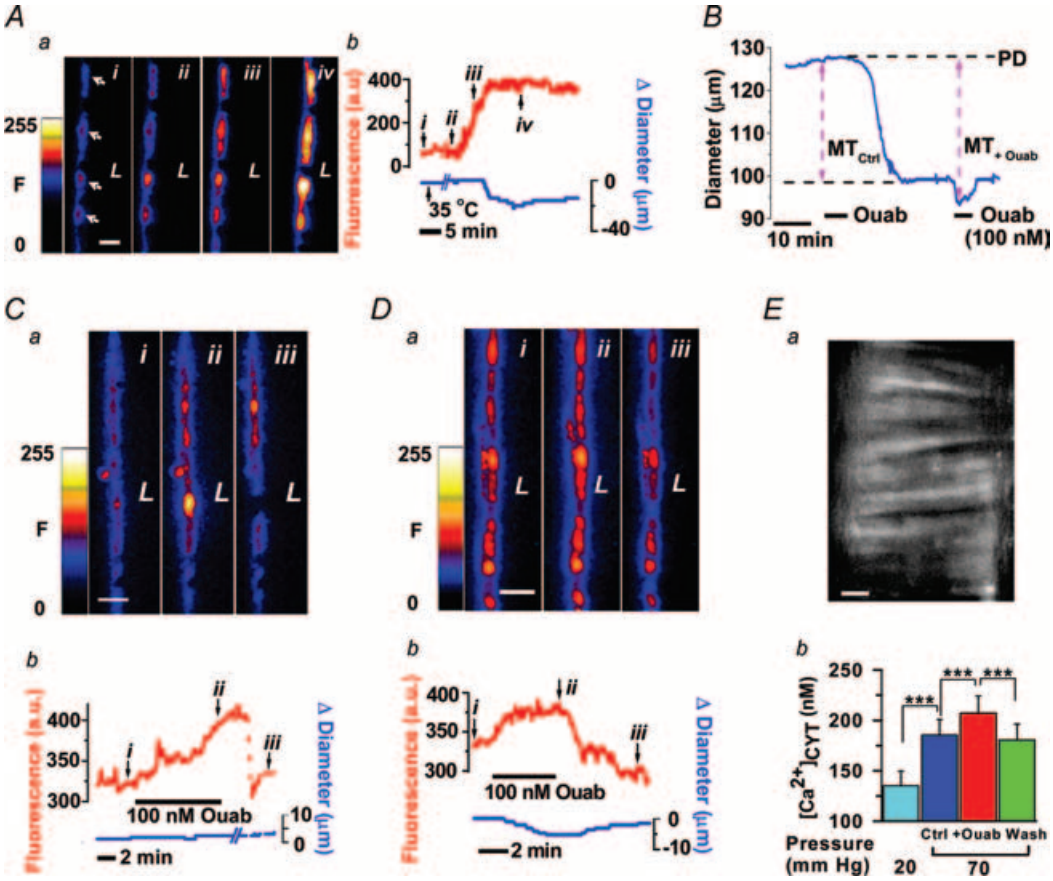

Figure 2. Influence of 100 nM ouabain on $[Ca^{2+}]_{cyt}$ and myogenic tone in WT mouse arteries
Aa, fluo-4 pseudocolor images from a representative artery wall cross-section captured at the times (*i–iv*) indicated in graph *b* when the artery was pressurized to 70 mmHg and warmed (at the arrow) to 35°C; L, lumen. *Ab*, simultaneous changes in diameter (Δ diameter = 2 × wall displacement) and $[Ca^{2+}]_{cyt}$ (i.e. average fluorescence in arbitrary units, a.u.) in the artery in panel *a* ($n = 13$). *B*, effects of 100 nM ouabain (Ouab) on the diameter of a representative artery before and after development of myogenic tone (MT) in the absence of fluo-4 ($n = 4$). Ouabain was applied during the periods indicated by the bars at the bottom of the graph. An intraluminal pressure of 70 mmHg was used to generate myogenic tone in these and all subsequent experiments, unless otherwise noted. PD, passive diameter; MT_{Ctrl}, control myogenic tone; MT_{+Ouab}, myogenic tone during exposure to ouabain. *Ca* and *Da*, fluo-4 pseudocolor images captured at the times (*i–iii*) indicated in graphs before (*Ca*) and after (*Da*) generation of myogenic tone. *Cb* and *Db*, $[Ca^{2+}]_{cyt}$ and diameter changes in representative arteries during exposure to 100 nM ouabain before (*C*; $n = 7$) and after (*D*; $n = 8$) development of myogenic tone. *Ea*, tangential image of a representative fura-2-loaded artery wall at 70 mmHg showing individual fluorescent myocytes orientated horizontally (the long axis of the artery is orientated vertically). *Eb*, average $[Ca^{2+}]_{cyt}$ in individual myocytes at 20 mmHg, and before, during and after treatment with 100 nM ouabain at 70 mmHg ($n = 22$ myocytes from three arteries). ***$P < 0.001$. Scale bars: 20 μm (*A* and *E*) or 10 μm (*C* and *D*).

the ouabain-induced constriction of internal diameter is ~0.45 μm per 1 nM rise in $[Ca^{2+}]_{cyt}$. Therefore, when Ca^{2+} sensitivity is high and $[Ca^{2+}]_{cyt}$ is already above contraction threshold in small arteries with myogenic tone, small changes in $[Ca^{2+}]_{cyt}$ should significantly affect vessel diameter.

These findings are comparable with published data from somewhat larger (~200 μm passive diameter) pressurized rat cerebral arteries with myogenic tone (Knot & Nelson, 1998): $[Ca^{2+}]_{cyt}$ was ~200 nM at 60 mmHg and 37°C, and K^+ depolarization constricted the arteries by ~1.05 μm per 1 nM increase in $[Ca^{2+}]_{cyt}$. Such small, but highly significant ouabain-induced increases in $[Ca^{2+}]_{cyt}$ and myogenic tone (Figs 2*B–E* and 3*A*) have profound physiological implications. Blood flow through small arteries is governed by Poiseuille's law (Berne & Levy, 2001), and resistance to flow, *R*, is inversely proportional to the fourth power of the internal radius, *r* (i.e. $R \propto 1/r^4$). For example, an ouabain-induced constriction from 85 to 75 μm internal diameter would be expected to increase *R* by 68% and markedly elevate BP.

Na^+ pump α2 subunits are the low-dose ouabain receptor

To elucidate the mechanism of action of low-dose ouabain on myogenic tone, it is important to identify the high affinity ouabain receptor. A dose of 10 nM ouabain also raised $[Ca^{2+}]_{cyt}$ (Fig. 3*Aa*), and the accompanying increase in myogenic tone (Fig. 3*Ab* and *B*) approached the maximal effect of 100 nM ouabain (Figs 2*B* and 3*D*). The relationship between the ouabain dose and the increase in myogenic tone was biphasic, with a plateau between 10 and 1000 nM (Fig. 3*D*). The apparent EC_{50} was ~1.3 nM at the high affinity ouabain site. This effect must be mediated by Na^+ pumps with high ouabain affinity α2 subunits. Even though the α1:α2 ratio is 4:1 in mesenteric arteries (Fig. 3*E*; the ratio is ~2.3:1 in the aorta (Shelly *et al.* 2004; Staton *et al.* 2005)), in rodents, Na^+ pumps with α1 subunits have ~1000-fold lower affinity for ouabain (O'Brien *et al.* 1994; Blanco & Mercer, 1998).

The artery wall contains endothelial cells and neurones as well as myocytes, and all the cells have Na^+ pumps/ouabain receptors. To determine whether

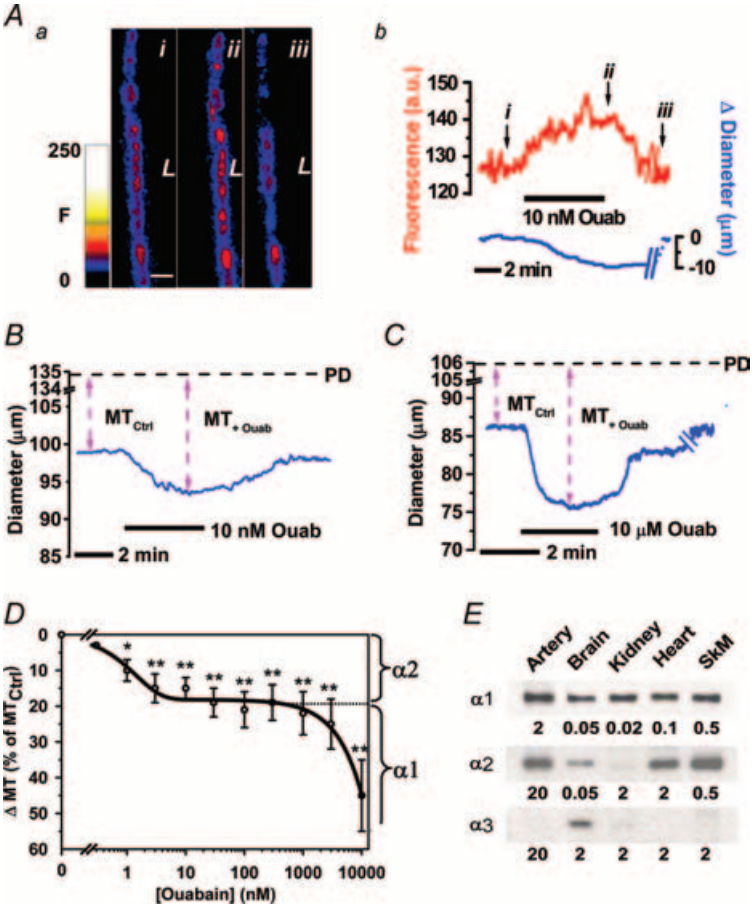

Figure 3. Effects of ouabain concentration on myogenic tone in WT arteries; roles of the Na^+ pump α1 and α2 isoforms
Aa, fluo-4 pseudocolor images from a representative artery captured at the times (*i–iii*) indicated in graph *b*. *Ab,* simultaneous $[Ca^{2+}]_{cyt}$ and diameter changes during exposure to 10 nM ouabain in an artery (*Aa*) with myogenic tone ($n = 6$). Scale bar, 10 μm. *B,* effect of 10 nM ouabain on myogenic tone in a representative artery in the absence of fluo-4. Ouabain (10 nM) increased myogenic tone (MT) from 21 ± 2% to 25 ± 2% of passive diameter (PD) ($n = 7$; $P < 0.01$). *C*, effect of 10 μM ouabain on myogenic tone in a representative artery in the absence of fluo-4. Ouabain (10 μM) increased myogenic tone (MT) from 23 ± 3% to 33 ± 4% of passive diameter (PD) ($n = 5$; $P < 0.01$). *D*, change in myogenic tone (ΔMT, as a percentage of control MT) graphed as a function of ouabain concentration ($n = 7$). Brackets at the right indicate the components of ΔMT that correspond to inhibition of the Na^+ pump high ouabain affinity α2 and low ouabain affinity α1 isoforms, respectively. $^*P < 0.05$; $^{**}P < 0.01$ *versus* control (before ouabain); the value at 10 μM was significantly greater than at 100 nM or 1 μM ($P < 0.01$). *E,* immunoblots of Na^+ pump α subunit isoform (α1, α2 and α3) distribution in mouse mesenteric artery and other tissues. Numbers are micrograms of protein per lane. Since the skeletal muscle (SkM) α1:α2 ratio is ≈1:4 (He *et al.* 2001; Golovina *et al.* 2003), the normalized band densities (see Methods) indicate that mesenteric artery α1:α2 ≈ 4:1 and heart α1:α2 ≈ 6.3:1.

endothelial cells play a major role in the response to ouabain, the endothelium was denuded. This is indicated by the loss of ACh-induced vasodilatation in arteries constricted with 5 μM phenylephrine (PE; Fig. 4*A*, red). Then, following development of myogenic tone, 100 nM ouabain still augmented myogenic tone by $21 \pm 4\%$ ($n = 3$; Fig. 4*A*, green). Thus, the endothelium had little influence on the response to nanomolar concentrations of ouabain.

The possible contribution of ouabain-induced catecholamine release from sympathetic nerve terminals to the ouabain-induced vasoconstriction (Bagrov *et al.* 1995) was tested by blocking myocyte α-adrenoceptors. Phentolamine (1 μM) blocked 90% of the vasoconstriction induced by 5 μM PE (not shown) but had no influence on the 100 nM ouabain-induced vasoconstriction (Fig. 4*B*, red line *versus* control blue and green lines). Even 10 μM phentolamine, which abolished the response to 10 μM PE, had no effect (not shown). Thus, catecholamine release by sympathetic nerves apparently contributed little to the ouabain-induced increase in myogenic tone.

Inhibition of Na^+ pumps, which are electrogenic, might be expected to depolarize the myocytes and thereby trigger vasoconstriction (Haddy & Overbeck, 1976; but see Blaustein, 1981). However, 100 nM ouabain, which should block only about 20% of total Na^+ pumps (i.e. only those with $\alpha 2$ subunits), had negligible effect on the membrane potential of myocytes within intact mesenteric arteries (Fig. 4*C*). On average, 100 nM ouabain depolarized the myocytes by only 0.1 ± 0.5 mV ($n = 6$). As these experiments were performed on rat arteries, it is important to note that 100 nM ouabain increased myogenic tone from a $21 \pm 3\%$ to a $27 \pm 3\%$ constriction relative to passive diameter in rat mesenteric small arteries ($n = 5$; $P < 0.01$).

The influence of the external K^+ concentration ($[K^+]_o$) was tested in order to demonstrate that small, reversible changes in membrane potential could be detected. Unlike ouabain, elevating $[K^+]_o$ from 4.9 to 10 mM depolarized the arteries by about 4–5 mV (Fig. 4*C*). Indeed, a hyperpolarization might have been expected (Weston *et al.* 2002) because such small increases in $[K^+]_o$ often dilate small arteries (Emanuel *et al.* 1959), perhaps as a result of Na^+ pump activation (Weston *et al.* 2002). Nevertheless, the subject is controversial because some other investigators also have observed that a 5 mM rise in $[K^+]_o$ depolarizes small arteries (Quinn *et al.* 2000; Bratz *et al.* 2002).

Myogenic tone is increased in $\alpha 2^{+/-}$ mice

Figures 2–4 suggest that 10–100 nM ouabain raises $[Ca^{2+}]_{cyt}$ and increases myogenic tone by inhibiting myocyte Na^+ pumps with $\alpha 2$ subunits. Therefore we also studied myogenic tone in mesenteric small arteries from mice with a single null mutation in the gene that encodes either the $\alpha 1$ or $\alpha 2$ isoform of the Na^+ pump α subunit ($\alpha 1$ or $\alpha 2$ heterozygotes: $\alpha 1^{+/-}$ or $\alpha 2^{+/-}$; James *et al.* 1999). Arteries from the heterozygous mice expressed ~50% of normal $\alpha 1$ or $\alpha 2$, respectively (Fig. 5*A* and *B*) (James *et al.* 1999; Shelly *et al.* 2004). Thus, despite up-regulation of $\alpha 2$, total Na^+ pump expression was reduced by ~40% in $\alpha 1^{+/-}$ arteries (Fig. 5*A* and *B*). Nevertheless, $\alpha 1^{+/-}$ arteries generated the same amount of myogenic tone at 70 mmHg pressure, and the same response to 100 nM ouabain as did WT mouse arteries (Fig. 6*A*).

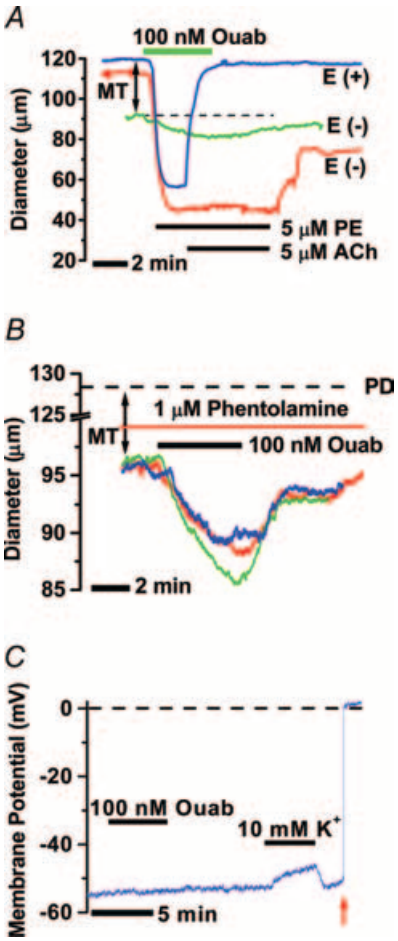

Figure 4. The effect of nanomolar concentrations of ouabain on myogenic tone is not mediated by the endothelium, catecholamine release or myocyte depolarization
A, effect of endothelium removal on ouabain's action in a representative artery. ACh-evoked endothelium-dependent relaxation of phenylephrine (PE) vasoconstriction (E(+); blue) is absent (red) after removing endothelium. Endothelium removal (E(−)) with an intraluminal air bubble (25–30 min) did not prevent the generation of myogenic tone (MT) or the effect of 100 nM ouabain on myogenic tone (green) ($n = 3$). *B*, effect of 100 nM ouabain on myogenic tone in a representative artery in the absence (before and after, blue and green, respectively) and presence (red) of 1 μM phentolamine ($n = 3$). *C*, effects of 100 nM ouabain and 10 mM K^+ on myocyte resting membrane potential (V_m) in a representative rat intact mesenteric small artery ($n = 6$). The electrode was withdrawn from the myocyte at the red arrow.

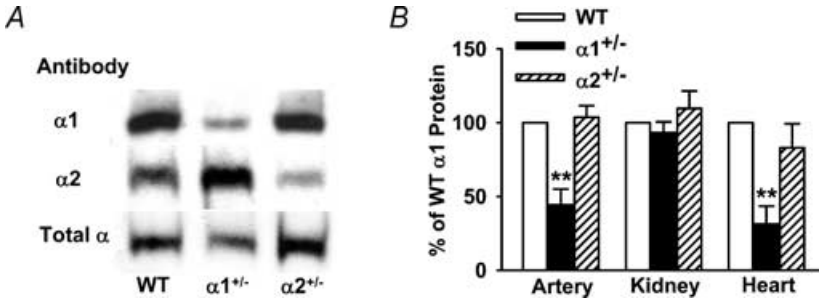

Figure 5. Na^+ pump α1 and α2 isoform expression in mesenteric arteries and other tissues from WT, $\alpha1^{+/-}$ and $\alpha2^{+/-}$ mice
A, representative immunoblots showing Na^+ pump α1, α2 and total α subunit expression levels in mesenteric arteries from WT, $\alpha1^{+/-}$ and $\alpha2^{+/-}$ mice. *B,* comparison of relative α1 expression (normalized data; see Methods) in mesenteric arteries, kidneys and hearts from WT, $\alpha1^{+/-}$ and $\alpha2^{+/-}$ mice ($n = 3$ for all bars). $^{**}P < 0.01$ *versus* WT and $\alpha2^{+/-}$.

In contrast, $\alpha2^{+/-}$ mouse arteries, in which total Na^+ pumps are reduced by only ∼10% (Fig. 5*A* and *B*), generated significantly more myogenic tone than did WT arteries, and the response to 100 nM ouabain was commensurately reduced (Fig. 6*A*). Indeed, 100 nM ouabain increased $[Ca^{2+}]_{cyt}$ (measured with fura-2) by only 7 ± 2 nM in $\alpha2^{+/-}$ artery myocytes ($n = 28$), *versus* 22 ± 5 nM in WT myocytes ($n = 22$; $P = 0.011$). Moreover, the myogenic responses to step increases in intraluminal pressure were augmented in both WT arteries treated with 100 nM ouabain and $\alpha2^{+/-}$ arteries (without ouabain) relative to myogenic responses in control WT arteries (Fig. 6*B*). Thus, low-dose ouabain increases myogenic responses and myogenic tone by inhibiting, selectively, Na^+ pumps with α2 subunits.

$\alpha2^{+/-}$ mice have high blood pressure

Maintained low nanomolar plasma ouabain induces a sustained hypertension in rodents that requires α2 Na^+ pumps with high affinity for ouabain (Dostanic *et al.* 2005). As reduced Na^+ pump α2 subunit expression mimics the effects of nanomolar ouabain on small arteries (Fig. 6*A* and *B*), we reasoned that $\alpha2^{+/-}$ mice might have elevated BP. Indeed, averaged mean BP (MBP) was significantly higher in $\alpha2^{+/-}$ mice than in WT mice under isofluorane anaesthesia (Fig. 7).

In striking contrast, $\alpha1^{+/-}$ mice, with far fewer α1 and total arterial Na^+ pumps (Fig. 5*A* and *B*), have normal BP (Fig. 7) as well as normal myogenic tone (Fig. 6*A*). Nevertheless, acute inhibition by 10 μM ouabain markedly elevates arterial myocyte $[Ca^{2+}]_{cyt}$ (not shown), constricts arteries (Fig. 6*A*) and has a large positive inotropic effect on hearts (James *et al.* 1999) from $\alpha1^{+/-}$ mice. Comparable effects of 10 μM ouabain are observed in WT mouse arteries (Figs 3 and 6*A*) and hearts (James *et al.* 1999). What, then, is the explanation for this difference between the effects of congenitally reduced α1 expression and acutely reduced α1 activity on myogenic tone, and for the normal BP in $\alpha1^{+/-}$ mice? The data imply that there is compensation for chronically reduced α1 activity. Up-regulation of α2 expression in the heart and arteries (Fig. 5*A*) of $\alpha1^{+/-}$ mice may provide some compensation even though α1 and α2 are localized to different plasma

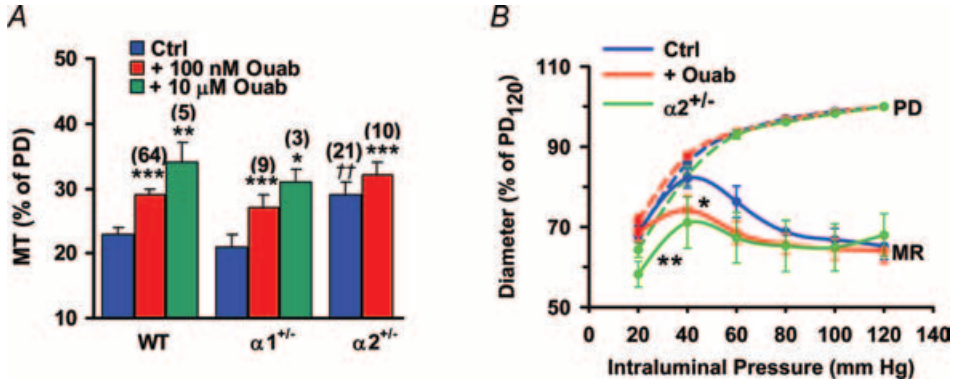

Figure 6. Effects of reduced Na^+ pump α1 and α2 isoform expression and ouabain on myogenic tone and myogenic reactivity
A, effects of ouabain on myogenic tone in WT, $\alpha1^{+/-}$ and $\alpha2^{+/-}$ mouse arteries. Myogenic tone (MT) is shown as a percentage of passive diameter (PD). $\dagger\dagger P < 0.05$ *versus* WT control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* genotype control (numbers of arteries in parentheses). *B*, effects of 100 nM ouabain (Ouab; red) and reduced Na^+ pump α2 expression ($\alpha2^{+/-}$; green) on passive diameter (PD, dashed lines) and myogenic reactivity to step changes in intraluminal pressure (MR, continuous lines). Blue lines, control (Ctrl) passive diameter and myogenic reactivity. Ordinate shows diameter as a percentage of passive diameter at 120 mmHg (PD120). $n = 7$ (WT), 6 (WT + ouabain) and 5 ($\alpha2^{+/-}$) arteries. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* WT MR; *P* values were determined by two-way ANOVA.

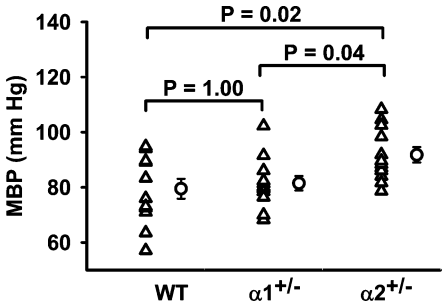

Figure 7. Effects of genetically reduced Na$^+$ pump α1 or α2 isoform expression on arterial blood pressure
Mean femoral artery blood pressure (MBP) in WT, $\alpha1^{+/-}$ and $\alpha2^{+/-}$ mice under 1.5% isofluorane anaesthesia. △, individual measurements for $n = 12$ mice in each group; ○, mean values. Mice were age-matched: WT = 113 ± 2, $\alpha1^{+/-}$ = 109 ± 4 and $\alpha2^{+/-}$ = 110 ± 4 days. *P* values were determined by one-way ANOVA.

membrane domains, are regulated differently and have different functions (Juhaszova & Blaustein, 1997). Other mechanisms also are likely to be involved, including reduced cell Na$^+$ permeability. Thus, arterial myocytes from $\alpha1^{+/-}$ mice may have relatively normal $[Na^+]_{cyt}$ and $[Ca^{2+}]_{cyt}$ despite the reduced α1 expression.

Alternatively, $\alpha1^{+/-}$ mice might be normotensive because they lose salt due to reduced expression of α1 in the kidneys. This is not the case, however, because renal α1 expression is not reduced in the $\alpha1^{+/-}$ mice, whereas cardiac and arterial α1 are reduced by ∼50% (Fig. 5*B*). As cardiac contractility is reduced in these mice (James *et al.* 1999), the BP might be normal because of a reduced CO. There is, however, no reason to expect a hypercontracted vasculature and an augmented TPR in $\alpha1^{+/-}$ mice, as myogenic tone is normal in isolated $\alpha1^{+/-}$ arteries (Fig. 6*A*).

Ouabain antagonists block ouabain's effect on tone

If the proposed sequence of events leading from ouabain to increased vascular tone (Fig. 1) is correct, it should be possible to interrupt this sequence with appropriate pharmacological tools. For example, PST 2238 and canrenone, known ouabain antagonists (Finotti & Palatini, 1981; Ferrari *et al.* 1998), should reduce ouabain's inhibition of Na$^+$ pump α2 subunits and augmentation of myogenic tone. Indeed, 5 μM PST 2238, an antihypertensive agent (Ferrari *et al.* 1998) derived from digitoxigenin (Quadri *et al.* 1997), abolished the effect of 100 nM ouabain on myogenic tone (Fig. 8*A* and *B*); prior application of PST prevented ouabain from augmenting myogenic tone (not shown). Canrenone (5 μM), a spironolactone metabolite with antihypertensive activity (Semplicini *et al.* 1995; Mantero & Lucarelli, 2000), was a partial antagonist (Fig. 8*B*). Neither agent affected control myogenic tone. Also, neither agent affected the increase in myogenic tone in $\alpha2^{+/-}$ arteries as, in this case, the reduced α2 activity was genetic and was not induced by ouabain.

NCX blockers inhibit effects of reduced α2 activity

One proposed mechanism by which ouabain increases myogenic tone is by reducing the Na$^+$ electrochemical gradient across the plasma membrane (PM) at PM–sarcoplasmic/endoplasmic reticulum (S/ER) junctions (Arnon *et al.* 2000; Golovina *et al.* 2003) where high ouabain affinity Na$^+$ pumps (Juhaszova & Blaustein, 1997; Shelly *et al.* 2004) and NCX1 (Juhaszova & Blaustein, 1997; Lencesova *et al.* 2004) are located. As in the heart (Hilgemann, 2004), the reduced Na$^+$ gradient should have

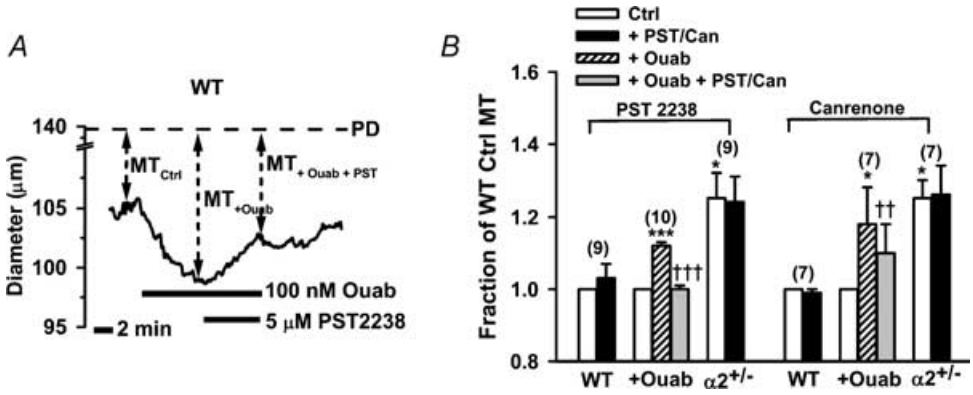

Figure 8. Effects of ouabain antagonists on myogenic tone augmented by 100 nM ouabain or by reduced α2 Na$^+$ pump expression
A, the action of 5 μM PST 2238 on 100 nM ouabain-augmented myogenic tone (MT) in a representative WT artery. *B,* summary of effects of 5 μM PST 2238 and 5 μM canrenone on control myogenic tone (WT Ctrl MT), and on myogenic tone augmented by 100 nM ouabain and by reduced α2 expression ($\alpha2^{+/-}$). **P* < 0.05, ****P* < 0.001 *versus* MT_{Ctrl} in WT arteries (MT_{Ctrl} is myogenic tone in the absence of ouabain). ††*P* < 0.01, †††*P* < 0.001 *versus* MT_{+Ouab} in WT arteries or MT_{Ctrl} in $\alpha2^{+/-}$ arteries (numbers of arteries in parentheses).

a profound effect: it should, via NCX1, raise $[Ca^{2+}]_{cyt}$ not only locally, but in the S/ER and bulk cytosol as well (Arnon *et al.* 2000; Golovina *et al.* 2003).

In the present study, 1 μM SEA0400 reduced control myogenic tone by ~10%, abolished the ouabain-induced increase in myogenic tone (Fig. 9*A* and *C*), and significantly attenuated the enhanced myogenic tone in arteries from $\alpha2^{+/-}$ mice (Fig. 9*B* and *C*). The latter effect contrasts with the absence of responses to PST 2238 and canrenone in $\alpha2^{+/-}$ mouse arteries (Fig. 8*B*). KB-R7943 (1 μM), another, less selective NCX inhibitor (Matsuda *et al.* 2001; Iwamoto *et al.* 2004*a*), had similar but less marked effects (Fig. 9*C*). These data cannot be explained by inhibition of L-type voltage-gated Ca^{2+} channels: Ca^{2+} entry through these channels accounts for most of control myogenic tone (Hill *et al.* 2001), but neither 1 μM SEA0400 nor KB-R7943 blocked the 75 mM K^+-induced, nifedipine-sensitive vasoconstriction in these arteries (not shown). Rather, the *in vitro* data (Fig. 9*C*) indicate that ~10% of control myogenic tone depends upon Ca^{2+} entry through NCX (presumably NCX1). Operation of a 3 Na^+:1 Ca^{2+} exchanger in the Ca^{2+} entry mode is not surprising because the myocyte membrane potential in pressurized arteries (about −45 to −55 mV; Knot & Nelson, 1998) may be positive to the NCX reversal potential (Blaustein & Lederer, 1999). Reduction of myogenic tone by SEA0400 and KB-R7943, rather than augmentation, when myogenic tone was amplified by reduced $\alpha2$ activity (Fig. 9*A*, *B* and *C*) also indicates that increased Ca^{2+} entry via NCX was responsible for the increased myogenic tone in ouabain-treated and $\alpha2^{+/-}$ arteries. Thus, arterial myocyte NCX1 mediates the rise in myogenic tone and elevation of BP induced by nanomolar ouabain and by reduced Na^+ pump $\alpha2$ subunit expression.

Discussion

$\alpha2$ Na^+pumps: long-term regulators of blood pressure in mice

This report reveals that low nanomolar ouabain augments myogenic reactivity and myogenic tone in small arteries by interacting specifically with arterial myocyte $\alpha2$ Na^+ pumps. These data are consistent with the recent report (Dostanic *et al.* 2005) that ouabain does not induce hypertension in mice with mutated, ouabain-resistant $\alpha2$ Na^+

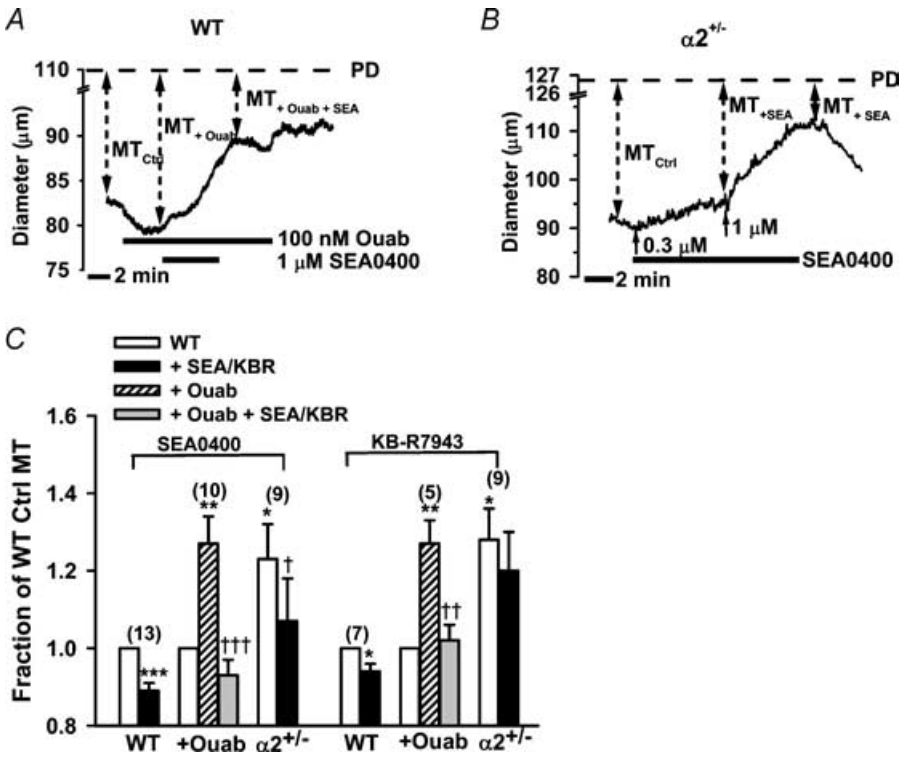

Figure 9. Effects of NCX blockers on myogenic tone augmented by 100 nM ouabain or by reduced $\alpha2$ Na^+ pump expression

A and *B*, representative experiments illustrating the actions of 1 μM SEA0400 on ouabain-augmented myogenic tone in a WT artery (*A*) and on myogenic tone in an $\alpha2^{+/-}$ artery (*B*). *C*, summary of effects of 1 μM SEA0400 and 1 μM KB-R7943 on control myogenic tone (WT Ctrl MT) and on myogenic tone augmented by 100 nM ouabain or by reduced $\alpha2$ expression ($\alpha2^{+/-}$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* MT_{Ctrl} in WT arteries. †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ *versus* MT_{+Ouab} in WT arteries or MT_{Ctrl} in $\alpha2^{+/-}$ arteries (numbers of arteries in parentheses).

pumps. The latter result demonstrates that interaction between ouabain and $\alpha 2$ is necessary for the induction of hypertension by ouabain, but this does not elucidate the mechanism.

Here, we employed ouabain concentrations approaching the EOLC levels in the circulation (~0.1–3.0 nM; e.g. Rossi *et al.* 1995), and we addressed the mechanism of ouabain's action in intact, small arteries. The data demonstrate that low-dose ouabain-induced vasoconstriction is a result of its direct action on the arterial myocytes; it is not due to effects on the endothelium, catecholamine release from the sympathetic neuroeffector cells, or myocyte depolarization. In the isolated arteries, brief (e.g. 5–15 min) treatment with low-dose ouabain mimics the effects of genetically reduced $\alpha 2$ expression on myogenic reactivity and myogenic tone. Clearly, there is little adaptation or compensation for reduction of $\alpha 2$ activity even over the lifetime of the mice. Also, BP is elevated in $\alpha 2^{+/-}$ mice, as it is in ouabain-treated rodents (Yuan *et al.* 1993; Manunta *et al.* 1994; Iwamoto *et al.* 2004*b*; Dostanic *et al.* 2005). Thus, the ouabain-induced increase in myogenic reactivity and myogenic tone, as well as the elevated BP, are primarily due to reduction of arterial myocyte $\alpha 2$ activity, *per se,* and not to other reported actions of ouabain (Santana *et al.* 1998; Aizman *et al.* 2001; Gao *et al.* 2002; Xie & Askari, 2002; Saunders & Scheiner-Bobis, 2004). Moreover, a preliminary report that overexpression of $\alpha 2$, but not $\alpha 1$, in mouse smooth muscle induces hypotension (Staton *et al.* 2005) is consistent with this view of the central role of arterial myocyte $\alpha 2$ Na^+ pump activity in regulating BP.

The evidence that BP is elevated in $\alpha 2^{+/-}$, but not $\alpha 1^{+/-}$ mice implies that reduced $\alpha 2$ activity is both necessary and sufficient to induce hypertension, even without ouabain. Thus, arterial myocyte $\alpha 2$ Na^+ pumps are a newly identified and long-sought long-term regulator of BP. The hypertension (and presumed increase in TPR) in the $\alpha 2^{+/-}$ mice are probably the consequence of the augmented myogenic reactivity and myogenic tone observed in the isolated $\alpha 2^{+/-}$ arteries. It is important to note, however, that these results do not preclude other, additional effects of EOLC in the pathogenesis of salt-dependent hypertension. For example, effects in the kidneys (in addition to vasoconstriction) may influence salt retention (Ferrandi *et al.* 2004), and in the kidneys and other tissues may contribute to target organ damage, possibly by promoting cell growth and proliferation (Aizman *et al.* 2001; Xie & Askari, 2002; Saunders & Scheiner-Bobis, 2004).

How does $\alpha 2$ activity influence blood pressure?

The increase in myogenic tone that results from reduced $\alpha 2$ Na^+ pump activity can be explained by a rise in $[Ca^{2+}]_{cyt}$ mediated by NCX1 (Iwamoto *et al.* 2004*b*), with which $\alpha 2$ Na^+ pumps are coupled both geographically (Juhaszova & Blaustein, 1997; Lencesova *et al.* 2004) and functionally (Arnon *et al.* 2000; Golovina *et al.* 2003). This view is supported by the pharmacological evidence that the augmented myogenic tone in arteries from $\alpha 2^{+/-}$ mice as well as the increased myogenic tone induced by nanomolar ouabain are blocked by SEA0400 and KB-R7943.

Salt-dependent hypertension was not explored in the present study. Indeed, the observation that the ouabain antagonists, PST 2238 and canrenone, did not influence the augmented myogenic tone in arteries from $\alpha 2^{+/-}$ mice implies that the $\alpha 2^{+/-}$ hypertension model is ouabain (and salt) independent, presumably because salt and ouabain act upstream (Fig. 1). Nevertheless, a preliminary report indicates that the development of deoxycorticosterone acetate (DOCA)-salt hypertension is accelerated in $\alpha 2^{+/-}$ (*versus* WT) mice (Staton *et al.* 2005). This is expected if, when there are already a reduced number of $\alpha 2$ Na^+ pumps, the DOCA and salt elevate plasma EOLC (Hamlyn *et al.* 1991; Rossi *et al.* 1995; Goto & Yamada, 2000).

In summary, these data are consistent with the recent report (Iwamoto *et al.* 2004*b*) that salt-dependent and ouabain-induced hypertension share the same downstream mechanism; namely, enhanced NCX1-mediated Ca^{2+} entry into arterial myocytes via NCX1 (Fig. 1). This is, presumably, the consequence of a reduced Na^+ concentration gradient across the plasma membrane in the vicinity of the $\alpha 2$ Na^+ pump and NCX1 clusters (Juhaszova & Blaustein, 1997; Arnon *et al.* 2000; Golovina *et al.* 2003; Lencesova *et al.* 2004).

Implications for human hypertension

The essential link between reduced $\alpha 2$ activity and ouabain-induced hypertension in rodents (Yuan *et al.* 1993; Manunta *et al.* 1994; Iwamoto *et al.* 2004*b*; Dostanic *et al.* 2005) has important implications for human hypertension. EOLC is elevated in rodents with salt-dependent hypertension (Hamlyn *et al.* 1991; Ferrandi *et al.* 1998; Takada *et al.* 1998). EOLC is also elevated in humans with mineralocorticoid hypertension (Rossi *et al.* 1995; Goto & Yamada, 2000) and in a large fraction of patients with essential hypertension (Rossi *et al.* 1995; Manunta *et al.* 1999; Pierdomenico *et al.* 2001; Goto & Yamada, 2000). It would be surprising if the mechanisms responsible for raising BP in salt-dependent hypertension are fundamentally different in rodents and humans. Nevertheless, the markedly different affinities of human and rodent $\alpha 1$ for ouabain seem puzzling. Human $\alpha 1$ has high affinity for ouabain, and nanomolar EOLC should inhibit human $\alpha 1$ as well as $\alpha 2$; thus, very different effects of EOLC might be expected in humans and rodents.

We observed, however, both normal myogenic tone in isolated $\alpha1^{+/-}$ arteries, and normal BP in $\alpha1^{+/-}$ mice, despite the markedly reduced $\alpha1$ activity in the arteries. Nevertheless, brief exposure to high-dose ($\geq1\ \mu$M) ouabain, which should inhibit rodent $\alpha1$ Na^+ pumps (O'Brien *et al.* 1994; Blanco & Mercer, 1998), induced profound vasoconstriction of the isolated, small arteries from $\alpha1^{+/-}$ mice as well as those from WT and $\alpha2^{+/-}$ mice. The implication is that the $\alpha1^{+/-}$ mice compensate for the chronically (genetically) reduced $\alpha1$ activity, and thereby avoid a rise in BP. This contrasts with the apparent absence of compensation for genetically reduced $\alpha2$ activity, mentioned above.

Perhaps in humans, too, if there is compensation for chronically EOLC-inhibited $\alpha1$ activity, functionally comparable with the reduced $\alpha1$ expression in $\alpha1^{+/-}$ mice, the dominant long-term effect of EOLC in humans may still be on arterial myocyte $\alpha2$. This would reconcile the dilemma about the proposed role of EOLC in human hypertension (Fig. 1). Indeed, such compensation for $\alpha1$ inhibition seems likely because this isoform is the 'housekeeper' that maintains the low global $[Na^+]_{cyt}$ (Golovina *et al.* 2003). Complete inhibition or knockout of rodent $\alpha2$ Na^+ pumps (only ~20% of total arterial Na^+ pumps) has minimal effect on global $[Na^+]_{cyt}$ (Golovina *et al.* 2003) and membrane potential (Fig. 4*C*). In contrast, acute inhibition of a significant fraction of the predominant $\alpha1$ Na^+ pumps (e.g. by micromolar ouabain concentrations) can be expected to elevate global $[Na^+]_{cyt}$ substantially (Golovina *et al.* 2003) and markedly depolarize the myocytes (Aalkjaer & Mulvany, 1985); this should induce profound vasoconstriction.

Antagonism of ouabain's effect

An intriguing feature of cardiotonic steroid action is the fact that *Strophanthus* steroids such as ouabain and dihydro-ouabain induce hypertension in rodents, whereas *Digitalis* steroids such as digoxin and digitoxin do not (Kimura *et al.* 2000; Manunta *et al.* 2001). This seems surprising because both classes of steroids inhibit the Na^+ pump and, as emphasized here, reduced $\alpha2$ Na^+ pump activity is necessary and sufficient for induction of hypertension in rodents. However, the fact that digoxin and digitoxin not only do not induce hypertension, but *lower* blood pressure in ouabain-hypertensive rats (Manunta *et al.* 2000) and even in human hypertensives (Abarquez, 1967), provides an important clue. This observation implies that the *Digitalis* steroids are partial Na^+ pump agonists (i.e. they are pump inhibitors) and partial antagonists (i.e. they block ouabain's inhibition of the Na^+ pump). Indeed, the digitoxigenin derivative, PST 2238, is a particularly interesting synthetic furane analogue of a *Digitalis* steroid because it has negligible agonist activity and strong ouabain-antagonist activity (Fig. 8) (Ferrari *et al.* 1998). The observation that PST 2238 antagonizes the low-dose ouabain-induced increase in myogenic tone, but has no effect on the augmented myogenic tone in arteries from $\alpha2^{+/-}$ mice, is consistent with this mechanism of action.

We conclude that there is now compelling evidence for the sequence of events illustrated in Fig. 1. EOLC, Na^+ pumps with $\alpha2$ subunits and NCX1 are key downstream components in the regulation of myocyte $[Ca^{2+}]_{cyt}$ and contractility, and long-term control of BP. This pathway (Fig. 1) provides several novel targets for antihypertensive therapy. These include the biosynthetic and secretory pathways of EOLC as well as Na^+ pumps with $\alpha2$ subunits and NCX1. Finally, by clarifying the mechanisms involved in salt-dependent hypertension, it should now be easier to elucidate the mechanisms that underlie the pathogenesis of other forms of essential hypertension.

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