Activation of c-SRC underlies the differential effects of ouabain and digoxin on Ca$^{2+}$ signaling in arterial smooth muscle cells

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Zulian A, Linde CI, Pulina MV, Baryshnikov SG, Papparella I, Hamlyn JM, Golovina VA. Activation of c-SRC underlies the differential effects of ouabain and digoxin on Ca$^{2+}$ signaling in arterial smooth muscle cells. Am J Physiol Cell Physiol 304: C324–C333, 2013. First published November 28, 2012; doi:10.1152/ajpcell.00337.2012.—Cardiotoxic steroids (CTS) of the strophanthus and digitalis families have opposing effects on long-term blood pressure (BP). This implies hitherto unrecognized divergent signaling pathways for these CTS. Prolonged ouabain treatment upregulates Ca$^{2+}$ entry via Na$^{+}$/Ca$^{2+}$ exchanger-1 (NCX1) and TRPC6 gene-encoded receptor-operated channels in mesenteric artery smooth muscle cells (ASMCs) in vivo and in vitro. Here, we test the effects of digoxin on Ca$^{2+}$ entry and signaling in ASMC. In contrast to ouabain treatment, the in vivo administration of digoxin (30 μg·kg$^{-1}$·day$^{-1}$ for 3 wk) did not raise BP and had no effect on resting cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{cyt}$) or phenylephrine-induced Ca$^{2+}$ signals in isolated ASMCs. Expression of transporters in the α2 Na$^{+}$ pump-NCX1-TRPC6 Ca$^{2+}$ signaling pathway was not altered in arteries from digoxin-treated rats. Uptregulation of α2 Na$^{+}$ pumps and a phosphorylation of the e-c-Src protein kinase (pY419-Src, ~4.5-fold) were observed in ASMCs from rats treated with ouabain but not digoxin. Moreover, in primary cultured ASMCs from normal rats, treatment with digoxin (100 nM, 72 h) did not upregulate NCX1 and TRPC6 but blocked the ouabain-induced upregulation of these transporters. Pretreatment of ASMCs with the c-Src inhibitor PP2 (1 μM; 4-amino-5-(4-chlorophenyl)-7-(t-butylypyrazolo[3,4-d]pyrimidine) but not its inactive analog eliminated the effect of ouabain on NCX1 and TRPC6 expression and ATP-induced Ca$^{2+}$ entry. Thus, in contrast to ouabain, the interaction of digoxin with α2 Na$^{+}$ pumps is unable to activate c-Src phosphorylation and upregulate the downstream NCX1-TRPC6 Ca$^{2+}$ signaling pathway in ASMCs. The inability of digoxin to upregulate c-Src may underlie its inability to raise long-term BP.

cardiotoxic steroids; Na$^{+}$ pumps; NCX1; TRPC proteins; receptor-operated Ca$^{2+}$ entry

Cardiotoxic steroids (CTS) have a long history in the treatment of heart failure and certain cardiac arrhythmias (3). Besides their role in regulation of cardiac function, endogenous CTS that are structurally related to the strophanthus family and that include ouabain are also implicated in control of salt metabolism and blood pressure (BP) (11, 49). Plasma levels of endogenous ouabain are significantly elevated in ~45% of patients with essential hypertension (48, 55) and in animals with several forms of salt-dependent hypertension (16, 21, 26). Moreover, in rodents, the prolonged administration of low doses of ouabain induces ouabain-induced hypertension (OH) (29, 30, 39, 40, 46, 61).

Endogenous ouabain influences arterial tone and peripheral vascular resistance (10, 12, 20) by activating Ca$^{2+}$ signaling pathways in arterial smooth muscle cells (ASMCs) (46). These “signature” pathways involve inhibition of high-affinity α2 Na$^{+}$ pumps and increased Ca$^{2+}$ entry via the Na$^{+}$/Ca$^{2+}$ exchanger-1 (NCX1), and TRPC gene-encoded receptor-operated channels (ROC5 and ROC6, respectively) (46). Rodent ASMCs express α2 Na$^{+}$ pumps that mostly modulate Ca$^{2+}$ homeostasis and Ca$^{2+}$ signaling (3). Prior work has shown that the expression of α2 Na$^{+}$ pumps, NCX1 and TRPC1 and TRPC6 is upregulated in ASMCs from OH rats (46). These transporters are involved in control of agonist-mediated vasoconstriction (51, 63), myogenic tone, and BP (25, 57, 62, 63). The arterial expression of NCX1 and TRPC6 is augmented in Milan hypertensive rats (64) and TRPC6 is upregulated in mineralocorticoid-salt hypertensive rats (4); both hypertension models are characterized by elevated plasma levels of endogenous ouabain (16, 21).

In contrast to ouabain, digoxin, which is also a specific Na$^{+}$ pump inhibitor with high affinity for α2 in rodents (42), does not raise BP in normal rats (39). Moreover, digoxin is an effective antihypertensive in the OH rat (24, 29, 39), and Digitalis preparations have antihypertensive actions in some patients with essential hypertension (1). As the opposing effects of these CTS on long-term BP are not explained by differences in their ability to inhibit α2 Na$^{+}$ pumps, the hypertensinogenic action of ouabain involves binding to α2 Na$^{+}$ pumps (14, 15, 37) followed by the specific triggering of a co-related signaling event.

The Na$^{+}$ pump also acts as a hormone receptor that can transduce the binding of circulating CTS into activation of intracellular protein kinases and Ca$^{2+}$ signaling (32, 35). Ouabain binding to Na$^{+}$ pumps activates the nonreceptor tyrosine kinase, c-Src, and mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinases (ERK1/2) (32). Members of the Src-kinase family can phosphorylate/regulate TRPC channels (22, 27, 54), while MAPKs regulate expression of NCX1 (59). Here, we tested the hypothesis that the opposing long-term effects of ouabain and digoxin on BP can be explained by their effects on arterial smooth muscle Ca$^{2+}$ signaling.

METHODS

Ethical approval. All experiments were carried out according to the guidelines of and were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

Ouabain- and digoxin-treated rats. Male 7- to 8-wk-old Sprague-Dawley rats (Charles River Laboratories, Frederick, MD) were maintained in an air-conditioned, temperature (23°C)-controlled facility with a 12:12-h light/dark cycle. Rats had free access to tap water and were fed standard rat chow (containing 0.5% wt/wt sodium and 1.1%
wt/wt potassium) ad libitum. Body weight was measured weekly. Rats were allowed to acclimatize to the facility and the procedures of BP measurements for 1 wk preceding actual data collection. The rats were randomized to three groups to receive ouabain, digoxin (both 30 \( \mu \)g kg\(^{-1}\)day\(^{-1} \)) in phosphate-buffered saline, or vehicle given subcutaneously, via Alzet model 2004 mini osmotic pumps (39). Systolic and mean BPs were recorded weekly by tail-cuff plethysmography using a commercial photoelectric system (model 29 Blood Pressure Meter/Amplifier; IITC, Woodland Hill, CA) and a device providing constant rates of cuff inflation and deflation. The average values for BP in each rat were obtained typically from five sequential cuff inflation-deflation cycles (46). Systolic (SBP) and mean (MBP) blood pressures were obtained by direct inspection of the recordings. Diastolic BPs were calculated from the standard blood pressure equation: 

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\text{DBP} = (3\text{MBP} - \text{SBP})/2.
\]

Dissection of arteries for immunoblotting. The superior mesenteric artery and aorta from euthanized rats were rapidly excised and placed in ice-cold Hank’s balanced salt solution (HBSS). The arteries were cleaned of fat and connective tissue, de-endothelialized, frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\) before protein extraction, as described (8).

Fresly dissociated ASMCs for Ca\(^{2+}\) imaging. Myocytes were isolated from rat mesenteric arteries, as described (8). The superior mesenteric artery was cleaned of fat and connective tissue, and digested in low-Ca\(^{2+}\) (0.05 mM) physiological salt solution (PSS) containing 2 mg/ml collagenase type XI, 0.16 mg/ml elastase type IV, and 2 mg/ml bovine serum album (BSA, fat free) for 35 min at 37°C. The PSS contained (in mM) 140 NaCl, 5.9 KCl, 1.2 NaH\(_2\)PO\(_4\), 5 NaHCO\(_3\), 1.4 MgCl\(_2\), 1.8 CaCl\(_2\), 11.5 glucose, and 10 HEPES (pH 7.4). After digestion, the tissue was washed three times with low-Ca\(^{2+}\) PSS at 4°C. A suspension of single cells was obtained by gently triturating the tissue with a fire-polished Pasteur pipette in low-Ca\(^{2+}\) PSS. Smooth muscle cells were differentiated by their characteristic elongated morphology. Dispersed cells were directly deposited on glass coverslips for fluorescence microscopy. ASMCs on coverslips were stained with fura-2-AM for 35 min at 20 –22°C, with 5% CO\(_2\)-95% air (8). Freshly dissociated ASMCs for Ca\(^{2+}\) imaging, Myocytes were incubated for 45 min at 37°C in Ca\(^{2+}\)-and Mg\(^{2+}\)-free HBSS containing 1 mg/ml collagenase, type 2. After the incubation, the adventitia was carefully stripped, the endothelium was removed, and segments of the muscle layer were stored overnight in Dulbecco’s modified Eagle’s medium (DMEM) at 37°C (17). The next morning, ASMCs in the remaining smooth muscle were dissociated by digestion for 35–40 min at 37°C in Ca\(^{2+}\)- and Mg\(^{2+}\)-free HBSS containing 1 mg/ml collagenase, type 2, and 0.5 mg/ml elastase, type IV. The dissociated cells were resuspended and plated on either 25-mm coverslips for use in fluorescent microscopy experiments or on 10-cm culture dishes for Western blot analysis. The plated cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) under a humidified atmosphere of 5% CO\(_2\)-95% air at 37°C. The medium was changed on days 4 and 7. In cultures in which the effects of ouabain and/or digoxin were tested, the standard growth medium was replaced by serum-free growth medium 24 h before the CTS were added. Experiments were performed on subconfluent cultures on days 8–9 in vitro if not indicated otherwise. The purity of ASMC cultures was verified by positive staining with smooth muscle-specific \(\alpha\)-actin (17). Most of the cells (>99.5%) were \(\alpha\)-actin-positive.

Calcium imaging. The cytosolic free Ca\(^{2+}\) concentration, [Ca\(^{2+}\)]\(_{cyt}\), was measured with fura-2 by using digital imaging (8). Primary cultured ASMCs were loaded with fura-2 by incubation for 35 min in culture medium containing 3.3 \(\mu\)M fura-2-AM (20–22°C, 5% CO\(_2\)-95% air). After dye loading, the coverslips were transferred to a tissue chamber mounted on a microscope stage, where cells were superfused for 15–20 min (35–36°C) with PSS to wash away extracellular dye. Cells were studied for 40–60 min during continuous superfusion with PSS (35°C).

The imaging system was designed around a Zeiss Axiovert 100 microscope (Carl Zeiss, Thornwood, NY). The dye-loaded cells were illuminated with a diffraction grating-based system (Polychrome V, TILL Photonics, Germany). Fluorescent images were recorded using a CoolSnap HQ2 CCD camera (Photometrics, Tucson, AZ). Image acquisition and analysis were performed with a MetaFluor/MetaMorph Imaging System (Molecular Devices, Downingtown, PA). [Ca\(^{2+}\)]\(_{cyt}\) was calculated from the ratio of fura-2 fluorescent emission (510 nm) with excitation at 380 and 360 nm (the isosbestic point), as described (8). Intracellular fura-2 was calibrated in situ in freshly dissociated and in primary cultured ASMCs (8). Intracellular Ca\(^{2+}\) measurements are shown as fura-2 340/380 excitation ratios with fluorescent emission at 510 nm (8).

Immunoblotting. Cultured ASMCs were harvested in PBS supplemented with protease inhibitor cocktail tablets and were pelleted (3,000 g, 4°C, 20 min). The cell pellet was resuspended in lysis buffer containing (in mM) 145 NaCl, 10 NaH\(_2\)PO\(_4\), 10 Na\(_2\)SO\(_4\), and 1% IGEPAL supplemented with protease inhibitor cocktail tablets. The suspension was centrifuged (5,000 g, 4°C, 30 min). The supernatant containing the extracted proteins was mixed with sodium dodecyl sulfate (SDS) buffer containing 5% 2-mercaptoethanol, and the proteins were separated by SDS-PAGE (8). Similar immunoblot analysis was performed on proteins extracted from the frozen arteries (8). Typically, superior mesenteric arteries from two to four rats were pooled and pulverized with a stainless steel mortar and pestle. Membrane proteins were solubilized in SDS buffer, and separated by SDS-PAGE as described (8). The following antibodies were used: rabbit polyclonal anti-TRPC6 (dilution 1:200) (Allomone Laboratories, Jerusalem, Israel); mouse monoclonal anti-NCX1 (dilution 1:500) (R3F1; Swant, Bellinzona, Switzerland); rabbit polyclonal anti-Na\(^+\)pump \(\alpha\)-subunit isoform (dilution 1:2,000) (gift of Dr. Thomas Pressley); rabbit polyclonal anti-Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-2-subunit isoform (dilution 1:750) (Millipore, Billerica, MA); rabbit polyclonal anti-phospho-Src family (Tyr418) (dilution 1:1,000) (Cell Signaling Technology, Danvers, MA); rabbit polyclonal anti-Src (dilution 1:1000) (Cell Signaling Technology, Danvers, MA). Gel loading was controlled with monoclonal anti-\(\beta\)-actin antibodies (dilution 1:10,000) (Sigma-Aldrich, St. Louis, MO) or monoclonal anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (dilution 1:5,000) (Abcam, Cambridge, MA). After being washed, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated IgG for 1 h at room temperature. The immune complexes on the membranes were detected by enhanced chemiluminescence-plus (Amersham Biosciences, Piscataway, NJ) and exposure to X-ray film (Eastman Kodak, Rochester, NY). Quantitative analysis of immunoblots was performed by using a Kodak DC120 digital camera and 1D Image Analysis Software (Eastman Kodak).
elastase (type IV), BSA, nifedipine, phenylephrine, penicillin G, and streptomycin were purchased from Sigma-Aldrich. Protease inhibitor cocktail tablets were obtained from Roche Applied Science (Indianapolis, IN). All other reagents were analytic grade or the highest purity available.

Statistical analysis. The numerical data presented in results are means ± SE from n single cells (1 value per cell). Immunoblots were repeated at least four to six times for each protein. The number of animals is presented where appropriate. Data from 6–18 rats were obtained for most protocols. Statistical significance was determined using Student’s paired or unpaired t-test, or two-way ANOVA, as appropriate. P < 0.05 was considered significant.

RESULTS

Comparison of chronic in vivo effects of digoxin and ouabain on BP and ASMC Ca 2+ signaling ex vivo. As shown in Fig. 1A, sustained hypertension was induced by the prolonged administration of ouabain but not digoxin. By the second week of the treatments, systolic BP increased only in the ouabain-infused group and remained elevated until the rats were euthanized at week 3 (137 ± 3 mmHg vs. vehicle 116 ± 4 mmHg, P < 0.05). Systolic BP in digoxin-treated (“digoxin”) rats was not significantly altered (113 ± 4 mmHg; P > 0.05). Diastolic BP was 91 ± 2, 86 ± 2, and 88 ± 2 mmHg, respectively, in ouabain-, digoxin-, and vehicle-treated rats (P < 0.05 ouabain treated vs. digoxin treated). Previous work using the same infusion protocol has shown that the plasma ouabain and digoxin levels are raised substantially in the CTS-infused groups, typically reaching 3–5 nM (39). These levels are well above the level of endogenous CTS, i.e., endogenous ouabain ∼0.5 nM and digoxin-like immunoreactivity <0.08 nM in the control rats (39).

Confirming a prior observation, the prolonged administration of ouabain induced significantly higher resting [Ca2+]cyt and phenylephrine (PE)-induced Ca2+ responses in freshly dissociated mesenteric artery myocytes vs. those from control rats (40). However, in contrast to ouabain, the prolonged administration of digoxin did not affect resting [Ca2+]cyt or PE-induced Ca2+ signals (Fig. 1, B–D). Accordingly, in all subsequent experiments with digoxin, ouabain was used in parallel as a positive control. Both the peak initial Ca2+ response, believed to be the result of inositol trisphosphate (InsP3)-mediated SR Ca2+ release, and the later sustained Ca2+ signal, mediated by Ca2+ entry through ROCs and/or SOCs, were not altered in ASMCs from digoxin-treated rats (Fig. 1, B–D). Superimposed records of the PE-induced Ca2+ response show that the integral of [Ca2+]cyt (area under the [Ca2+]cyt curve) in OH arterial myocytes was increased to 131 ± 4% of the area in the control (vehicle) ASMCs (n = 28 control cells; n = 36 OH cells, P < 0.05). The area under the [Ca2+]cyt curve in arterial myocytes from digoxin-treated rats was not significantly altered (103 ± 3%; P > 0.05).

Comparison of chronic in vivo digoxin and ouabain on ASMC α2 Na+ pumps and NCX1. Altered Ca2+ signaling in freshly dissociated mesenteric artery myocytes from OH rats is associated with upregulated expression of α2 Na+ pumps, NCX1 and TRPC1 and TRPC6. Moreover, α2 Na+ pumps and NCX1 mediate the effects of low-dose ouabain on smooth muscle Ca2+ signaling and vasoconstriction (15, 25, 62). However, as shown in Fig. 2, A and B, the expression of α2 Na+ pumps was not altered in mesenteric arteries from digoxin-treated rats in contrast to the ~2.5-fold α2 Na+ pump upregulation in arteries from ouabain-treated rats. Further, the expression of α1 Na+ pumps, which have very low affinity for ouabain (43) and digoxin (42) in rodents, was not altered in arteries from ouabain- or digoxin-treated rats (Fig. 2, C and D). In addition, expression of NCX1 also was not altered in arteries from digoxin-treated rats (Fig. 2, E and F), while it was markedly upregulated (~5–6-fold) in ouabain-treated rats (46).

Effect of chronic in vivo digoxin and ouabain on ROC-mediated Ca2+ entry and TRPC6 expression in ASMC. To determine whether ROC-mediated Ca2+ entry contributes to differential PE-induced Ca2+ responses in arterial myocytes from “digoxin” and ouabain-treated rats (Fig. 1, B and D), freshly dissociated ASMCs were stimulated with the cell-permeable diacylglycerol (DAG) analog OAG. OAG opens TRPC3 and TRPC6 channels in a protein kinase C-independent manner (23). Ba2+ was used as the charge carrier because

![Fig. 1. Blood pressure and calcium homeostasis in mesenteric artery myocytes from control normotensive (NT), ouabain-hypertensive (OH), and digoxin-treated (Digox) rats. A: systemic blood pressure in control (NT), OH, and digoxin-treated rats. Normal male rats were infused with either vehicle (control), ouabain (30 μg·kg−1·day−1), or digoxin (30 μg·kg−1·day−1) for 3 wk. Representative groups of rats (n = 9 each) are shown. *P < 0.05 vs. vehicle-treated rats (ANOVA). B: phenylephrine (PE)-induced Ca2+ transients in freshly dissociated arterial smooth muscle cells (ASMCs) from control (NT), OH, and “digoxin” rats. Representative records show the time course of cytosolic free Ca2+ concentration ([Ca2+]cyt) changes induced by 1 μM PE. C: summarized data showing resting [Ca2+]cyt (C; 112 control, 99 OH and 102 “digoxin” cells) and peak PE-induced Ca2+ transient (D; 57 control, 52 OH and 53 “digoxin” cells) in ASMCs from 9 control, 9 OH, and 9 digoxin-treated rats. ***P < 0.001 vs. ASMCs from control rats.](http://ajpcell.physiology.org/ by 10.1183/22293330.2017.901325)
**Fig. 2.** Expression of Na\(^+\) pump α1- and α2-subunit isoforms and NCX1 in arterial myocytes from control NT, OH, and digoxin-treated rats. Western blot analysis of Na\(^+\) pump α2 (A, B), α1 subunits (C, D), and NCX1 (E, F) protein expression in smooth muscle cell membranes from mesenteric arteries of control (NT), OH rats, and digoxin-treated (Digox) rats. A, C, and E show representative blots. All lanes were loaded with 30 µg of membrane protein. Summary data (B, D, and F) are normalized to the amount of β-actin and are expressed as means ± SE from 4 (B), 4 (D), and 6 (F) immunoblots (total of 18 rats). ***P < 0.001 vs. ASMCs from control NT rats.

SOCs have high Ca\(^{2+}\) selectivity and, unlike ROCs, are virtually impermeable to other alkaline-earth cations, such as Ba\(^{2+}\). Further, Ba\(^{2+}\) is not transported by SERCA or PM Ca\(^{2+}\) pumps (31). In the presence of extracellular Ba\(^{2+}\), 80 µM OAG induced significantly larger elevations of cytosolic Ba\(^{2+}\) (fura-2 340/380 nm ratio) in myocytes from OH rats than in those from digoxin-treated or control rats (Fig. 3, A and B). To eliminate the contribution of voltage-gated Ca\(^{2+}\) channels to OAG-induced Ba\(^{2+}\) entry, all solutions contained 10 µM nifedipine (46). The differential effects of in vivo administration of these CTS on ROC-mediated Ca\(^{2+}\) entry were associated with significant (~6-fold) upregulation of TRPC6 in deendothelialized mesenteric artery from OH but not from digoxin rats (Fig. 3, C and D). TRPC6 is an obligatory component of endogenous ROCs in a variety of cell types including vascular myocytes (23, 46). Expression of TRPC3 was not significantly affected in ASMCs from ouabain- (46) or digoxin-treated rats (not shown). The expression of TRPC1 and TRPC5, which are believed to form subunits of SOCs (7), was not altered in ASMCs from digoxin-treated rats (not shown), but expression of TRPC1 was upregulated in mesenteric arteries from ouabain-treated rats (46). TRPC4 is not expressed in rat mesenteric arteries (8). The results show that digoxin, in contrast to ouabain, is unable to upregulate the α2-NCX1-TRPC6 (ROC) signaling pathway.

Differential regulation of phosphorylated Src and ERK1/2 in ASMCs from digoxin- and ouabain-treated rats. Binding of ouabain to Na\(^+\) pumps activates c-Src and stimulates various pathways including ERK1/2 and members of the MAP kinase family of serine/threonine kinases (32). Src and MAP kinase families play a key role in ASMC signaling events (44) and regulation of arteriolar contractility (53). The chronic in vivo administration of ouabain and digoxin had different effects on arterial expression and phosphorylation of endogenous c-Src

**Fig. 3.** 1-Oleoyl-2-acetyl-sn-glycerol (OAG)-induced [receptor-operated channel (ROC)-mediated] Ba\(^{2+}\) entry and TRPC6 protein expression in freshly dissociated ASMCs from control NT, OH, and digoxin-treated rats. A: representative records show the time course of the Fura-2 fluorescence ratio (F\(_{340}/F_{380}\)) signals induced by 80 µM OAG in freshly dissociated ASMCs from control, OH, and digoxin-treated rats. Extracellular Ca\(^{2+}\) was replaced by 1 mM Ba\(^{2+}\) during the period indicated on the graph. Nifedipine (10 µM) was applied 10 min before the trace shown and was maintained throughout the experiment. B: summarized data show the OAG-induced, ROC-mediated Ba\(^{2+}\) entry in 28 control, 22 OH, and 25 digoxin-treated rat mesenteric ASMCs. Each bar corresponds to data from 6 rats. C and D. Western blot analysis of TRPC6 expression in ASMCs from control (NT), OH, and digoxin-treated (Digox) rats. C: representative immunoblot (30 µg protein/lane). D: summarized data from 8 immunoblots (total of 18 rats) are normalized to β-actin. ***P < 0.001 vs. control ASMCs.
and ERK1/2 (Fig. 4, A–E). In these studies, activation of c-Src was assessed with an anti-phospho-Src antibody that detects c-Src phosphorylation/activation at Tyr-418 (pY-418 Src). As shown in Fig. 4, A and B, pY418-Src was upregulated ~4- to 5-fold in mesenteric arteries from ouabain- but not digoxin-treated rats. The antibody detected multiple bands between 49 and 60 kDa, consistent with the presence of multiple Src family members in ASMCs (2). All bands were greatly increased in the ouabain-treated group. In contrast, the phosphorylated form of ERK1/2 was downregulated (by ~2-fold) in ouabain- but not digoxin-treated rats (Fig. 4, A and D). Expression of constitutive c-Src and ERK1/2 was not altered by any treatment (Fig. 4, A, C, E).

In vitro replication of the actions of in vivo ouabain and digoxin. As shown in Fig. 5, treatment of cultured ASMCs from normal rats with ouabain for 72 h enhanced NCX1 and TRPC6 expression and Ca²⁺ signaling. Parallel digoxin treatment of primary cultured rat mesenteric artery myocytes had no effect on the expression of these transporters. However, digoxin completely blocked the stimulatory effect of ouabain on NCX1 (Fig. 5, A and B) and TRPC6 expression (Fig. 5, C and D). The ouabain-evoked changes in NCX1 and TRPC6 expression were reflected in elevated resting [Ca²⁺]cyt and augmented PE-induced Ca²⁺ responses, whereas digoxin was inactive (Fig. 6). Similar results (not shown) were obtained with ATP-evoked responses; treatment with 100 nM ouabain for 72 h increased ATP-induced Ca²⁺ responses (905 ± 77 vs. 582 ± 84 nM in control untreated ASMCs; n = 49, P < 0.001). Thus the lack of effects of in vivo digoxin administration on arterial Ca²⁺ signaling observed in Fig. 1, B–D, is confirmed ex vivo with cultured ASMCs. Superimposed records of the PE-induced Ca²⁺ response show that the integral of the rise of [Ca²⁺]cyt (area under the [Ca²⁺]cyt curve) in ouabain-treated ASMCs was increased to 138 ± 3% of the area in control untreated ASMCs (n = 39 control ASMCs; n = 43 ouabain-treated ASMCs, P < 0.005). The area under the [Ca²⁺]cyt curve in the digoxin-treated ASMCs was not altered (101 ± 2%; n = 49 cells, P > 0.05). Thus the in vitro effects of prolonged ouabain or digoxin treatment on arterial myocytes mimic the in vivo changes.
The major new results are as follows. 1) Ouabain-treated ASMC. Ouabain treatment increased ATP-

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turally related and commonly used CTS, digoxin, to upregulate

2

mechanisms involved in the differential regulation of arterial

DISCUSSION

The present report describes the first study of some key

Involvement of Src kinases as a determinant of ouabain- and digoxin-induced responses in primary cultured rat ASMCs. Pretreatment of ASMCs with the Src kinase inhibitor PP2 (1 μM) blocked the ability of ouabain to upregulate NCX1 and TRPC6 expression (Fig. 7, A–D); pretreatment of ASMCs with PP3 (1 μM), an inactive analog of PP2, did not prevent the ouabain-induced upregulation of either NCX1 (Fig. 7, E and F) or TRPC6 (Fig. 7, G and H).

The ouabain-evoked changes in Ca2+ transporter expression were also reflected in altered Ca2+ handling following PP2 pretreatment. Figure 8A illustrates the protocol: it shows the time course of ATP-induced changes in [Ca2+]cyt in the absence and presence of extracellular Ca2+ in control and ouabain-treated ASMC. Ouabain treatment increased ATP-induced SR Ca2+ release (the initial rise in [Ca2+]cyt in Fig. 8A) only marginally (Fig. 8B) but augmented the secondary rise in Ca2+ (∼55%) associated with Ca2+ entry via SOCs and ROCs (Fig. 8, A and C). Exposure of cultured control ASMCs to 1 μM PP2 for 72 h did not affect the ATP-induced Ca2+ release but modestly increased extracellular Ca2+ entry (Fig. 8, B and C). However, PP2 treatment eliminated the effect of ouabain on Ca2+ influx (Fig. 8C).

4)

transporter expression and signaling in arterial myocytes. 4) The differential signaling evoked by two different ligands acting within a common receptor indicates that, like many seven transmembrane G-protein coupled receptors (GPCRs), the α2 Na+ pump isofrom in ASMCs exhibits a profound functional ligand bias.

Overall, the results provide the first explanation for, and are highly consistent with, the view that the hypertensive effect of ouabain in vivo involves activation of α2 Na+ pump-Src kinase signaling and that this event is critical for the subsequent upregulation of Ca2+ entry mediated by NCX1 and TRPC6 (ROC). These findings also provide the first molecular explanation for the paradoxical antihypertensive effects of digoxin in salt-sensitive and ouabain hypertensive rats (24, 39), and the antihypertensive effect of digitalis preparations in some hypertensive patients (1).

Digoxin and ouabain differentially regulate Ca2+ homeostasis in rat ASMCs. There is accumulating evidence that endogenous Na+ pump inhibitors play an important role in cardiovascular, renal, and other disorders (5). Among the endogenous CTS, endogenous ouabain is elevated in a large portion of patients with never-treated essential hypertension (38). Moreover, prolonged ouabain infusions, that generally replicate the elevated plasma levels observed in patients, induce hypertension (30, 39, 40, 46, 61). However, patients with heart failure have long been treated with digitalis preparations (38) without the side effect of hypertension. If hypertension is not a recognized side-effect of digitalis treatment, why would ouabain raise BP when all the classic CTS are well-documented Na+ pump inhibitors (6, 60)? Further, ouabain and digoxin have comparable binding affinity for rodent α2 (Kd of 0.26 and 0.15 μM, respectively) (42). Indeed, in rodents, both ouabain and digoxin, when acutely applied in nanomolar doses, increase the vascular tone of resistance arteries (9, 34, 47, 62). In humans also, both digoxin (41) and ouabain (50) share a short-term vasopressor action. Moreover, in humans digoxin augments pressor responses to norepinephrine and angiotensin (19).

While the acute vascular effects of ouabain and digoxin appear to be generally indistinguishable in humans and rodents, and consistent with the classical view of their mechanism of action, there has been no explanation for the ability of prolonged infusions of ouabain to induce hypertension in rodents. Similarly, the absence of long-term pressor activity with digoxin is also an enigma (24, 29, 39). The present results are thus of relevance as they show that ouabain and digoxin have very different effects on ASMC Ca2+ signaling that are mediated by specific Na+ and Ca2+ transport proteins (Figs. 1–3). Indeed, the ability of ouabain to upregulate Na+ pump α2-subunits, NCX1, and TRPC6 proteins in the ASMCs from ouabain-infused rats stands in stark contrast to the lack of any observed effect in the digoxin-treated rats. The first component in the signaling pathway affected by ouabain is the high ouabain affinity α2 Na+ pump. The CTS binding site is critical for the hypertensinogenic activity of ouabain; mutation of the α2 binding site to a CTS resistant form prevents ouabain- and adrenocorticotropic hormone (ACTH)-induced hypertension (14, 15, 37).

Of critical relevance, digoxin is unable to replicate the effects of ouabain on Ca2+ signaling not only in vivo but also in vitro. This obviates uncertainties associated with differences in whole body volume of distribution, clearance, and metabo-
lism as an explanation for their disparate signaling effects in excised arteries. Exposure of isolated ASM to nanomolar digoxin also had no effect on NCX1 and TRPC6 protein expression in marked contrast to ouabain (Fig. 5). Digoxin, however, was able to block the upregulating effect of ouabain (100 nM, 72 h) on NCX1 and TRPC6 expression in cultured rat ASMCs. Exposure of isolated ASMC to nanomolar ouabain (72 h) in the absence and presence of 1 μM PP2. E–H; PP3 (1 μM) did not prevent the upregulating effect of ouabain (100 nM, 72 h) on NCX1 and TRPC6 expression in cultured rat ASMCs. A, C, E, and G show representative blots. All lanes were loaded with 30 μg of membrane protein. Summary data (B, D, F, and H) are normalized to the amount of β-actin and are expressed as means ± SE from 8 (B), 7 (D), 4 (F), and 4 (H) immunoblots. *P < 0.05 and ***p < 0.001 vs. control ASMCs.

Elimination of the ouabain effects on NCX1 and TRPC6 expression by digoxin normalized Ca$^{2+}$ entry and arterial tone. Indeed, digoxin (Fig. 1A) and digitoxin do not induce hypertension in rats, and, moreover, reverse ouabain-dependent hypertension (39). The remarkable differences in the long-term effects of these two Na$^+$ pump inhibitors suggest that their common receptor, the α2 Na$^+$ pump, exhibits “biased” functional selectivity (28). In such a system, ligand binding evokes a series of signals, some common to all ligands (i.e., inhibition of Na$^+$ pumps by CTS) and other signals that are sensitive to specific structural features of the ligand (e.g., ouabain-evoked...
Ca\textsuperscript{2+} signaling). One of the best documented examples of ligand biased functional responses are the seven transmembrane G protein-coupled receptors which generate different intracellular signals that depend upon discrete and often small structural variations in the ligand (13). To our knowledge, the present results are the first molecular evidence indicating that the Na\textsuperscript{+} pump behaves in a manner analogous to GPCRs in this regard. The functional basis for the ligand bias is that Na\textsuperscript{+} pump-bound ouabain and digoxin likely interact with multiple amino acid residues in the binding site, while only certain interactions unique to bound ouabain trigger c-Src activation. In this regard, the spatial configuration of the binding site residues and hence ligand interaction is likely modified by long range interaction with intracellular binding partners. Detailed information on the identity of Na\textsuperscript{+} pump binding partners, and especially those that might trigger activation of Src, is not available in ASMCs. Further, the binding partners may vary among different cell types so that the signaling effects of ouabain and digoxin may be similar in some systems and not others and may even be \(\alpha\)-isoform-specific. For example, both ouabain and digoxin, when acutely applied at high doses, activated c-Src and inhibited protein synthesis in human cancer cell lines (56). Thus no evidence for biased CTS receptors (likely \(\alpha\)1 Na\textsuperscript{+} pumps) was observed in that study.

\textit{c-Src mediates the differential action of ouabain and digoxin on NCX1 and TRPC6 expression and Ca\textsuperscript{2+} signaling in rat ASMCs.} A key observation in this study is that pY418-Src is upregulated in mesenteric arteries from ouabain- but not digoxin-treated rats (Fig. 4, A and B) and that pretreatment with the c-Src inhibitor PP2 prevents ouabain from upregulating NCX1 and TRPC6 (Fig. 7, A–D) and ATP-induced Ca\textsuperscript{2+} influx (Fig. 8C). These results show that the ouabain-induced enhancement of NCX1 and TRPC6 expression reflects prior ouabain/Na\textsuperscript{+} pump mediated Src activation, i.e., c-Src is a key upstream regulator of the Na\textsuperscript{+} and Ca\textsuperscript{2+} transporters and Ca\textsuperscript{2+} signaling in ASMCs. Furthermore, the aforementioned data show that c-Src can be activated by chronic in vivo ouabain treatment at concentrations as low as \(~3\) nM (Fig. 4, A and B), overlapping the plasma level of ouabain in infused rats (39). The potent effects of ouabain on c-Src phosphorylation indicates this in vivo effect is mediated by the ouabain-binding \(\alpha\)2, but not the \(\alpha\)1 catalytic subunit because the latter is ouabain-insensitive in rodents.

In many cell types, ouabain activates c-Src, induces tyrosine phosphorylation of the epidermal growth factor receptor, and activates the Ras/ERK1/2 cascade (32, 49). Surprisingly, we did not observe activation of ERK1/2 in mesenteric arteries from OH rats, although c-Src was upregulated (Fig. 4, A, B, and D). In contrast, the phosphorylation of ERK1/2 was downregulated in OH rats and was unaffected in arteries from digoxin-treated rats (Fig. 4, A and D). The lack of ERK1/2 activation in OH rats may be explained, in part, by specificity in the \(\alpha\) isoforms that affect the degree of Na\textsuperscript{+} pump-mediated signaling to ERK1/2 (45). Indeed, Pierre and colleagues (45) demonstrated that \(\alpha\)2, in contrast to other \(\alpha\) subunits, does not support the ouabain-dependent ERK1/2 activation. The down-regulation of ERK1/2 phosphorylation in OH rats (Fig. 4, A and D) occurs through a pathway(s) which remains to be determined.

Once bound to Na\textsuperscript{+} pumps, the steps that link ouabain with c-Src activation and that trigger upregulation of the NCX1-TRPC6 (ROC) signaling pathway remain unclear. Studies of the high ouabain affinity \(\alpha\)1 Na\textsuperscript{+} pumps (33) of pig renal epithelial cells (32, 52) had suggested that ouabain-induced signaling results from a direct interaction of the \(\alpha\)1 subunit with Src protein kinases. Other recent work indicates that the ability of ouabain to activate c-Src is not mediated via direct contact between the kinase and Na\textsuperscript{+} pump subunits (36).

Src kinases can interact with TRPC channels and are involved in their regulation (22, 27, 54). For instance, Src phosphorylates TRPC3 at Y226 and formation of phospho-Y226 is essential for TRPC3 activation (27). Tyrosine phosphorylation by Src family protein-tyrosine kinases also regulates TRPC6 channel activity (22). Furthermore, TRPC6 acti-

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**Fig. 8. PP2 blocks the effect of 100 nM ouabain treatment (72 h) on ATP-evoked (SOC/ROC-mediated) Ca\textsuperscript{2+} entry in cultured rat ASMCs.** 

A: representative records showing the time course of [Ca\textsuperscript{2+}]\textsubscript{cyt} changes induced by 10 \muM ATP in Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-containing solutions in control untreated ASMC (blue line) and arterial myocytes treated with 100 nM ouabain for 72 h (red line). Nifedipine (10 \muM) was added 10 min before the records shown and was maintained throughout the experiments. B, C: summarized data showing the mean peak amplitudes \pm SE of ATP-induced Ca\textsuperscript{2+} release (B) and Ca\textsuperscript{2+} influx (C), measured from the basal ("resting") [Ca\textsuperscript{2+}]\textsubscript{cyt} \(\Delta\)([Ca\textsuperscript{2+}]\textsubscript{cyt} in nM); blue bars = control ASMCs, red bars = ouabain-treated ASMCs, cyan bars = PP2-treated ASMCs, and magenta bars = PP2 + ouabain-treated ASMCs. Ca\textsuperscript{2+} release data are from 89 control myocytes, 107 ouabain-treated, 151 PP2-treated, and 159 PP2 + ouabain-treated ASMCs. ATP-induced Ca\textsuperscript{2+} influx was studied in 90 control myocytes, 62 ouabain-treated, 153 PP2-treated, and 156 PP2 + ouabain-treated ASMCs. *P < 0.05 and ***P < 0.001 vs. control ASMCs.
vation by a receptor-tyrosine kinase-PLCγ pathway (triggered by epidermal growth factor) is inhibited by PP2 (22). Acute application of PP2 or genistein (a general tyrosine kinase inhibitor) also blocks Ca\(^{2+}\) entry via TRPC3 gene-encoded ROCs (27, 54). In this general context, it is not surprising that inhibition of c-Src activation prevents ouabain-induced up-regulation of TRPC6 (Fig. 7, C and D) and eliminates effect of ouabain on the ATP-induced Ca\(^{2+}\) influx (Fig. 8C). However, the exact relevance of the c-Src-TRPC6 interaction in the present context is not clear; we have previously shown that even in the presence of ouabain, the evoked upregulation of TRPC6 is absent when NCX1 upregulation is prevented (46). Under such conditions, c-Src should be active. Thus the c-Src-TRPC6 interaction may be important for channel activity, but by itself it is not sufficient to upregulate TRPC6 expression in these cells.

Finally, in view of all the aforementioned results, it seems unlikely that the different effects of ouabain and digoxin are associated with their ability to cross cell membranes. Like digoxin, ouabagenin (another Strophanthus steroid) crosses the plasma membranes fairly readily but retains the marked hypertensive activity of ouabain (39).

In summary, our results show that c-Src activation is a critical point of divergence that underlies the differential effects of ouabain and digoxin on the NCX1/TRPC6 Ca\(^{2+}\) signaling pathway in ASMCs. These findings represent further molecular evidence that ouabain-like prohypertensinogenic CTS have hormone-like signaling activity while other commonly used CTS that bind tightly to the same biased Na\(^{+}\) pump α2 subunit receptors not only lack this ability, but can act as antagonists with powerful BP lowering effects.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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