Ouabain modulation of endothelial calcium signaling in descending vasa recta

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Ouabain modulation of endothelial calcium signaling in descending vasa recta. Am J Physiol Renal Physiol 291: F761–F769, 2006. First published April 4, 2006; doi:10.1152/ajprenal.00326.2005.—Using fura 2-loaded vessels, we tested whether ouabain modulates endothelial cytoplasmic calcium concentration ([Ca2+]CYT) in rat descending vasa recta (DVR). Over a broad range between 10–10 and 10–4 M, ouabain elicited biphasic peak and plateau [Ca2+]CYT elevations. Blockade of voltage-gated Ca2+ entry with nifedipine did not affect the response to ouabain mitigating against a role for myo-endothelial gap junctions. Reduction of extracellular Na+mitigates against a role for myo-endothelial gap junctions. Reduction of extracellular Na+ concentration ([Na+]o) or Na+/Ca2+ exchanger (NCX) inhibition with SEA-0400 (10–5 M) elevated [Ca2+]CYT, supporting a role for NCX in the setting of basal [Ca2+]CYT. SEA-0400 abolished the [Ca2+]CYT response to ouabain implicating NCX as a mediator. The transient peak phase of [Ca2+]CYT elevation that followed either ouabain or reduction of [Na+]o, was abolished by 2-aminooxydiphenylborate (5 × 10–5 M). Cation channel blockade with La3+ (10 μM) or SKF-96365 (10 μM) also attenuated the ouabain-induced [Ca2+]CYT response. Ouabain pretreatment increased the [Ca2+]CYT elevation elicited by bradykinin (10–7 M). We conclude that inhibition of ouabain-sensitive Na+/K+ ATPase enhances DVR endothelial Ca2+ store loading and modulates [Ca2+]CYT signaling through mechanisms that involve NCX, Ca2+ release, and cation channel activation.

kidney; medulla; fura 2; SEA-0400; bradykinin; 2-aminooxydiphenyl borate

OUABAIN IS A CARDIOTONIC STEROID that inhibits plasmalemmal Na+/K+ ATPase by binding to its α-subunit. Ouabain, or a closely related analog [ouabain-like factor (OLF)], is endogenously produced by the adrenal glands and circulates systemically in low concentrations (8, 19, 21). The binding site for ouabain, the “ouabain receptor,” is highly conserved in evolution implying an important functional role for OLF. The role of ouabain has been debated for decades. A hypothesis is that inhibition of Na+ export from the cell by ouabain raises subplasmalemmal Na+ concentration, secondarily inhibiting Ca2+ export by Na+/Ca2+ exchange (NCX). One important effect of the decrease in Ca2+ export may be to augment loading of Ca2+ into endoplasmic/sarcoplasmic reticulum (ER/SR) stores. Through that putative mechanism, ouabain has been shown to augment agonist-induced cytoplasmic Ca2+ concentration ([Ca2+]CYT) transients and intensify vasoconstriction (2). In addition to such effects, mediated through NCX inhibition, elegant experiments by Xie and colleagues (50, 51) have shown that ouabain binding to Na+ pumps leads to downstream signaling events that may include activation of phospholipase C (PLC) and inositol tris-phosphate (InsP3) generation. In contrast to effects on smooth muscle and cardiac myocytes, the role of ouabain to modulate microvascular endothelial [Ca2+]CYT has not been as thoroughly explored.

Descending vasa recta (DVR) are 15-μm-diameter branches of juxtamedullary effenter arterioles that carry blood flow to the renal medulla. They are lined by a continuous endothelium and surrounded by smooth muscle pericytes that impart contractile function (36, 38). Agonists such as acetylcholine and bradykinin (BK) elevate endothelial [Ca2+]CYT to release vasodilators and limit DVR vasoconstriction (12, 37, 38, 43). Freshly isolated DVR are an attractive model to study endothelial [Ca2+]CYT responses in an intact microvessel preparation because the Ca2+-sensitive fluorophore fura-2 loads preferentially into the endothelium, sparing the pericytes (37). In this study, we exploited that feature to test the hypothesis that, as in smooth muscle and neurons, ouabain modulates DVR endothelial [Ca2+]CYT. Our results verify that ouabain, over a broad range of concentrations, increases basal [Ca2+]CYT in a biphasic manner. Na+/Ca2+ exchanger (NCX) and other pathways participate in the response. NCX blockade with SEA-0400, inositol trisphosphate receptor (InsP3R) blockade with 2-aminooxydiphenyl borate (2-APB), and nonselective cation channel blockade with La3+ or SKF-96365 interfere with the actions of ouabain. Finally, prolonged ouabain pretreatment increased the magnitude of [Ca2+]CYT elevation induced by BK suggesting enhancement of store loading of Ca2+ in DVR endothelium. These results imply that OLF may operate through complex signaling pathways to modulate vasoactivity in the renal medulla.

METHODS

Isolation of DVR. Investigations involving animal use described herein were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland. Kidneys were harvested from Sprague-Dawley rats (70–150 g; Harlan Sprague Dawley, Indianapolis, IN). Before nephrectomy, the rats were deeply anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection. Kidney slices were placed in dissection buffer and maintained at 4°C. The buffer used for dissection and superfusion of DVR contained (in mM) 140 NaCl, 10 Na-acetate, 5 KCl, 1.2 MgSO4, 1.2 NaHPO4, 5 HEPES, 5 d-glucose, 5 l-alanine, 0.1 l-arginine, and 1 CaCl2. The pH was adjusted to 7.55 at room temperature to yield a pH of ~7.4 at 37°C. Horizontal kidney slices were digested for 15–18 min in DMEM media containing Liberase Blendzyme 1 (Roche Boehringer Mannheim, 0.56 U Collagenase + Dispase in 0.7 ml DMEM) at 37°C. Individual DVR were dissected from the outer medulla and transferred to a heated chamber fitted to the stage of a Nikon Diaphot inverted microscope. The vessels were immobilized on glass pipettes.

Measurement of endothelial [Ca2+]CYT. DVR were loaded with fura 2-AM (5 μM) added to the bath at 37°C for 15 min. We
previously showed that fura 2 preferentially loads into the endothelial cells, yielding little fluorescent signal from pericytes (37). The vessels were visualized with a Nikon Fluor ×40 (numerical aperture 1.3) oil immersion objective. For measurement of \([\text{Ca}^{2+}]_{\text{CYT}}\) with fura 2, vessels were excited at 350 and 380 nm. Excitation frequencies were selected with a computer-controlled monochromator (PTI). A photon-counting photomultiplier assembly was fitted to the microscope and used to detect fluorescent emission from the probe. Fluorescent emissions were isolated using a 510WB-40 (Omega optical) filter. Background-subtracted fluorescence emission ratios \((R_{350}/380})\) were converted to \([\text{Ca}^{2+}]_{\text{CYT}}\) assuming a dissociation constant for fura 2 of 224 nM. \(R_{\text{min}}\) and \(R_{\text{max}}\) were measured in vessels exposed to 10⁻⁵ M ionomycin with 0 CaCl₂ and 5 × 10⁻⁴ M EGTA, or 5 × 10⁻³ M CaCl₂, respectively (37).

Membrane potential measurement. To obtain electrical access for membrane potential recording, we used perforated patches formed on endothelia. The abluminal surface of DVR endothelia was exposed by collagenase treatment and removal of pericytes. The electrode solution was (in mmol/l): 120 kasparsartate, 20 KCl, 10 NaCl, 10 HEPES, pH 7.2 and nystatin (100 μg/ml, 0.1% DMSO). The extracellular solution was physiological saline (PSS; in mmol/l): 155 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, and 10 glucose, pH 7.4. Membrane potential recordings were performed in current clamp mode \((I = 0)\) at a sampling rate of 10 Hz. The methods for endothelial exposure, patch-clamp recording, and junction potential correction have been extensively described (42).

Reagents. Stock solutions of SEA-0400 (2-{4-[(2,5-difluorophenyl) methoxy]phenoxy}-5-ethoxyaniline; Calbiochem, 10⁻⁴ M), nifedipine (Sigma, 10⁻² M), and 2-APB (Calbiochem, 10⁻² M) were prepared in DMSO. SKF-96365 (1-[[β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride, 10⁻² M) and BK (Sigma, 10⁻⁴ M) were dissolved in water and stored at –20°C. Ouabain (Sigma) was dissolved in dissection buffer at 10⁻⁴ M and stored at –20°C. Fura 2-AM (Molecular Probes, Eugene, OR) was stored frozen in anhydrous DMSO. Aliquots of reagents were thawed for dilution daily, and excess reagents were discarded at the end of each day.

Statistical analysis. Data in the text and figures are reported as means ± SE. The significance of differences was evaluated with SigmaStat 3.11 (Systat Software, Point Richmond, CA) using parametric or nonparametric tests as appropriate for the data. Comparisons between two groups were performed with Student’s \(t\)-test (paired or unpaired, as appropriate). Comparisons between multiple groups employed ANOVA or repeated-measures ANOVA. Post hoc comparisons were performed using Tukey’s or Holm-Sidak tests. \(P < 0.05\) was used to reject the null hypothesis.

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Fig. 1. Descending vasa recta (DVR) endothelial cytosolic Ca²⁺ concentration \([\text{Ca}^{2+}]_{\text{CYT}}\) changes evoked by ouabain. Vessels were exposed to log molar increasing concentrations of ouabain from 10⁻¹⁰ to 10⁻⁶ M \((n = 9)\). At each concentration, ouabain was introduced for 5 min and then removed for 5 min before introduction of the next ouabain concentration. A: representative experiment shows background-subtracted fluorescent ratios. B: peak (filled bars) and plateau (open bars, at 5 min) \([\text{Ca}^{2+}]_{\text{CYT}}\) expressed as % elevations from baseline. C: representative experiment from a separate series during exposure to ouabain at 10⁻⁶, 10⁻⁵, and 10⁻⁴ M \((n = 7)\). D: peak (filled bars) and plateau (open bars, at 5 min) \([\text{Ca}^{2+}]_{\text{CYT}}\) expressed as % elevations from baseline, means ± SE \((*P < 0.05)\). E: areas under the \([\text{Ca}^{2+}]_{\text{CYT}}\) response curves in the presence of ouabain (5 min, nM × s, data are means ± SE, \(*P < 0.05)\).
Results

Modulation of DVR endothelial \([\text{Ca}^{2+}]_{\text{CYT}}\) by ouabain. We first tested whether inhibition of ouabain-sensitive Na\(^+\)-K\(^+\)-ATPase affects basal endothelial \([\text{Ca}^{2+}]_{\text{CYT}}\). Baseline DVR endothelial \([\text{Ca}^{2+}]_{\text{CYT}}\) was typically 50–100 nM (Fig. 1) as previously reported (37, 41, 46). Exposure to incremental concentrations of ouabain between 0.1 nM and 1 \(\mu\)M led to increases in \([\text{Ca}^{2+}]_{\text{CYT}}\). Changes in \([\text{Ca}^{2+}]_{\text{CYT}}\) were reversible when ouabain was removed from the bath. The magnitudes of the \([\text{Ca}^{2+}]_{\text{CYT}}\) increases in peak and plateau phases of the response were similar from 10 to 10 \(\text{nM}\). Ouabain was removed from the bath. The magnitudes of the changes in \([\text{Ca}^{2+}]_{\text{CYT}}\) were 8.2 vs. 155.9, 75.3 vs. 12,864, 32.9 nM, area under curve; 6,447 ± 1,801 vs. 12,664 ± 2,603 nM × s, \(n = 7\), \(P < 0.05\). These data suggest that the effect of inhibition of low- and high-affinity Na\(^+\)-K\(^+\)-ATPase isoforms on \([\text{Ca}^{2+}]_{\text{CYT}}\) of DVR endothelia is additive.

Ouabain can depolarize cells by inhibiting the electrogenic exchange of 2K\(^+\) for 3Na\(^+\) by Na\(^+\)-K\(^+\)-ATPase. Thus secondary stimulation of Ca\(^{2+}\) influx via voltage-operated Ca\(^{2+}\) channels (VOCa) might occur into adjacent DVR pericytes upon ouabain application. Myo-endothelial gap junctions are preserved in this preparation and we have shown that DVR pericytes express nifedipine-sensitive VOCA (52). In view of that, we considered that ouabain might elevate endothelial \([\text{Ca}^{2+}]_{\text{CYT}}\) by increasing the influx of Ca\(^{2+}\) into pericytes followed by secondary transport of Ca\(^{2+}\) to the endothelium via gap junctions. To test that possibility, DVR were pretreated with nifedipine (10 \(\text{nm}\) and then exposed to ouabain (10 \(\text{nM}\)). Nifedipine did not inhibit ouabain-evoked \([\text{Ca}^{2+}]_{\text{CYT}}\) transients (Fig. 2A).

It is generally accepted that endothelial cells do not express VOCA (32); however, for completeness, we tested whether nanomolar ouabain depolarizes DVR endothelia. Endothelial membrane potential averaged 53 ± 5 mV at baseline and was not affected by exposure to 10 nM ouabain (Fig. 2B). Thus the depolarization that would be required for putative VOCA activation in endothelium did not occur. From these data, we infer that neither flux of Ca\(^{2+}\) across myoendothelial gap junctions nor voltage-gated Ca\(^{2+}\) entry into endothelial cells could account for ouabain-induced endothelial \([\text{Ca}^{2+}]_{\text{CYT}}\) transients.

Fig. 3. Effect of removal of Ca\(^{2+}\) from the bath on ouabain-evoked DVR endothelial \([\text{Ca}^{2+}]_{\text{CYT}}\) transients. Ouabain was introduced or sham exchange was performed 1 min after elimination of Ca\(^{2+}\) from the bath. A: representative recording of a single experiment shows background-subtracted fluorescent ratios. Arrows indicate time points corresponding to comparisons in B. B: bars show \([\text{Ca}^{2+}]_{\text{CYT}}\) measurements immediately before removal of Ca\(^{2+}\) (0 min in 1 mM Ca\(^{2+}\), arrow 1), 1 min after removal of Ca\(^{2+}\) (1 min in 0 mM Ca\(^{2+}\), arrow 2), and 1 min after ouabain or vehicle exposure (2 min in 0 mM Ca\(^{2+}\), arrow 3). Control, filled bars; ouabain, open bars; \(n = 4\) each. Data are means ± SE.

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Role of NCX in the ouabain-induced endothelial calcium transients. We hypothesized that, as in other cell types, ouabain inhibition of the α2-, α3-subunit sodium pumps might elevate [Na+] in the vicinity of the NCX thereby reducing clearance of Ca2+ from the endothelium. We first tested whether NCX activity can modulate [Ca2+]CYT of DVR endothelia by lowering extracellular sodium ([Na+]o) or calcium concentration ([Ca2+]o). As shown in Fig. 3, removal of Ca2+ from the bath rapidly reduced [Ca2+]CYT and eliminated ouabain (10⁻⁸ M)-induced [Ca2+]CYT transients. Given that other effects such as ER/SR store depletion and alteration of Ca2+ export via Ca2+-ATPase might accompany incubation of cells in 0 Ca2+ bath, we also tested the effect of lowering [Na+]o. Stepwise reduction of [Na+]o elicited incremental elevations of [Ca2+]CYT (Fig. 4), the magnitude of which was similar to that previously observed in aortic myocytes (3). Interestingly, similar to the effects of ouabain, reduction of [Na+]o yielded biphasic [Ca2+]CYT transients with peaks followed by persistent plateau elevations. These data favor a role for participation of NCX in the setting of basal [Ca2+]CYT.

To further establish a role for NCX, we examined baseline [Ca2+]CYT and ouabain-induced [Ca2+]CYT responses during NCX blockade with SEA-0400 (10⁻⁶ M), an inhibitor that is respected for its specificity to block NCX1 isoforms (24, 29, 47). Like reduction of [Na+]o, pharmacological inhibition of NCX increased baseline [Ca2+]CYT (control, 71.7 ± 5.1 nM, vs. SEA-0400, 179 ± 40.9 nM, n = 6 each, after 5 min at arrow 1, Fig. 5A). Furthermore, 5-min pretreatment with SEA-0400 almost completely abolished ouabain-induced [Ca2+]CYT elevations (control; from 72 ± 5.1 to 424 ± 69.8 nM, vs. SEA-0400; from 179 ± 40.9 to 215 ± 28.2 nM, P < 0.05, at arrow 2, Fig. 5B).

2-APB and cation channel blockade inhibits ouabain-evoked calcium transients. Hypothetically, inhibition of NCX could explain the ability of ouabain to elevate [Ca2+]CYT without invoking a need for participation of other Ca2+ transport pathways. The biphasic “peak and plateau” [Ca2+]CYT responses shown in Figs. 1–5, however, raise the question of release of Ca2+ from internal stores and influx of Ca2+ from the extracellular space via store-operated nonspecific cation channels. Given that ouabain enhances storage of Ca2+ in some cells (18) and has recently been shown to signal through PLC and InsP3 in renal epithelial (LLC-PK1) cells (50, 51), we tested other pathways. A selective blocker of InsP3R-mediated Ca2+ release from stores does not exist; however, 2-APB (5 × 10⁻⁵ M) blocks InsP3R along with store-operated Ca2+ channels (10, 11). As shown in Fig. 6, 2-APB decreased baseline endothelial [Ca2+]CYT (control; 106 ± 13.9, n = 7 vs. 2-APB; 62.4 ± 7.5 nM, n = 8). Exposure to ouabain in the presence of 2-APB led to a subdued [Ca2+]CYT response with a minimal, transient elevation (compare Figs. 1 and 6; control; from 106 ± 13.9 to 285 ± 28.3, vs. 2-APB; from 62.4 ± 7.5 to 86.7 ± 9.3 nM).

If InsP3 generation occurs in DVR endothelia exposed to ouabain, secondary activation of Ca2+ influx via nonspecific cation channels might also result from cellular store depletion. We tested for participation of such pathways by examining ouabain responses in the presence of SKF-96365 (10 μM) and La3+ (10 μM), agents that are known to block mechano- sensi-
Nonselective cation channels conduct both Na\(^+\) and Ca\(^{2+}\) into the cytoplasm. Reduction of extracellular Na\(^+\) might affect Ca\(^{2+}\) entry, independent of NCX, by reducing competition between those cations for ion channel selectivity filter(s) (35, 39). Alternately, cation channels might be activated during [Na\(^+\)]\(_o\) reduction through NCX inhibition that leads to Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) and store depletion. To test those possibilities, we performed experiments in which [Na\(^+\)]\(_o\) was reduced from 150 to 125 mM in the presence of La\(^{3+}\) (10 \(\mu\)M). DVR endothelial [Ca\(^{2+}\)]\(_{CYT}\) increased from 45 ± 9 to 221 ± 34 nM (Fig. 8, B and C) showing that block of La\(^{3+}\)-sensitive cation channels does not have significant effect on the response (compare with Fig. 8A).

**Ouabain enhancement of BK-evoked endothelial calcium transients.** It has been proposed that ouabain-induced inhibition of Ca\(^{2+}\) export leads to ER/SR store loading that favors enhancement of [Ca\(^{2+}\)]\(_{CYT}\) release by agonists (1, 2, 7). BK induces large peak-phase DVR endothelial [Ca\(^{2+}\)]\(_{CYT}\) transients attributable to ER/SR store release (37, 46). We therefore tested whether [Ca\(^{2+}\)]\(_{CYT}\) responses to BK are enhanced by ouabain. BK (10\(^{-7}\) M)-evoked [Ca\(^{2+}\)]\(_{CYT}\) elevation was augmented by prolonged (10 min) pretreatment with ouabain at a concentration (5 \(\times\) 10\(^{-5}\) M) that should affect all Na\(^+\)-K\(^+\)-ATPase isoforms (Fig. 9, A and B, area under the curve, 40,001 ± 8,765, \(n= 7\) vs. 100,159 ± 21,263 nM \(\times\) s, \(n= 7\), \(P<0.05\)). Basal [Ca\(^{2+}\)]\(_{CYT}\) was higher during ouabain exposure. When DVR were pretreated with ouabain at low concentration (10 \(\mu\)M) selective for \(\alpha_2/\alpha_3\) Na\(^+\)-K\(^+\)-ATPase, similar augmentation of the response to BK was observed (26,336 ± 3,883, \(n= 7\) vs. 44,808 ± 8,360 nM \(\times\) s, \(n= 7\), \(P<0.05\), Fig. 9, C and D).

**DISCUSSION**

Four isoforms of the Na\(^+\)-K\(^+\)-ATPase catalytic subunit (\(\alpha_1-\alpha_4\)) are expressed in mammalian cells. The \(\alpha_1\) catalytic subunit with low ouabain affinity is the dominant isoform that maintains transcellular Na\(^+\) and K\(^+\) concentration gradients and mediates salt reabsorption in the kidney. Circulating OLF is present in concentrations that are too low to affect the \(\alpha_1\)-isoform in rodents. One hypothesis proposes that the effect of ouabain to modulate [Ca\(^{2+}\)]\(_{CYT}\) signaling results from its inhibition of high-affinity \(\alpha_2\), \(\alpha_3\), or \(\alpha_4\)-isoforms (22, 30). Reduction of Na\(^+\) export then leads to an increase in localized [Na\(^+\)]\(_o\) (in the vicinity of NCX) that reduces or reverses the direction of NCX favoring reduction of Ca\(^{2+}\) export and augmentation of cellular ER/SR Ca\(^{2+}\) store loading (7, 8, 26). NCX and \(\alpha_2/\alpha_3\)-isoforms may be localized to discrete func-

![Fig. 6. Effect of 2-aminoethoxydiphenyl borate (2-APB) on ouabain-induced [Ca\(^{2+}\)]\(_{CYT}\) transients. Fura-2-loaded DVR were pretreated with 2-APB (5 \(\times\) 10\(^{-8}\) M) or vehicle before ouabain exposure. Baseline [Ca\(^{2+}\)]\(_{CYT}\) was lower in 2-APB and ouabain-evoked peak [Ca\(^{2+}\)]\(_{CYT}\) elevations were suppressed. Data are means ± SE.](http://ajprenal.physiology.org/)
cytoplasmic Ca\(^{2+}\) extrusion pumps (24, 29, 47). Nonetheless, some interpretational caution is in order because SEA-0400 has been shown to alter [Ca\(^{2+}\)]\(_{\text{CYT}}\) signaling in cells that lack NCX (40).

**Ouabain elicits calcium transients in the DVR endothelium.** Using fura 2-loaded DVR, we found that ouabain, at 0.1 nM-0.1 mM, affects basal DVR endothelial [Ca\(^{2+}\)]\(_{\text{CYT}}\) (Fig. 1). The lower end of that concentration range is similar to that of OLF in plasma (20) and is sufficient to affect \(\alpha_2/\alpha_3\) Na\(^{+}\)-K\(^{-}\)-ATPase in the rat. Several findings in Fig. 1 are of interest. First, ouabain modulates basal [Ca\(^{2+}\)]\(_{\text{CYT}}\) in this microvascular endothelium, an effect that is not uniformly present in all preparations (27, 28, 49). Because these studies were performed with fura-2, a probe that distributes diffusely into the cytoplasm, we conclude that ouabain-induced [Ca\(^{2+}\)]\(_{\text{CYT}}\) elevation occurs globally throughout the cells. It is possible that much greater effects on [Ca\(^{2+}\)]\(_{\text{CYT}}\) occur in the “junctional region” near the plasma membrane and NCX. Such a compartmental effect would not be delineated by fura 2 for two reasons. First, the affinity of fura 2 for Ca\(^{2+}\) is low; i.e., near-membrane Ca\(^{2+}\) binding to fura 2 might be saturated if junctional Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{JNT}}\)) concentrations are very high. Second, fura 2 fluorescence from the bulk cytoplasm probably overwhelms any emanations from the junctional cytoplasm.

A second, interesting feature of the response in Fig. 1 is that ouabain gives a biphasic [Ca\(^{2+}\)]\(_{\text{CYT}}\) change comprised of an early peak followed by a sustained plateau. Observation times in Fig. 1 were brief; however, plateau [Ca\(^{2+}\)]\(_{\text{CYT}}\) elevations are sustained for at least 10 min (data not shown). Finally, the response to ouabain is remarkably similar over a broad range of concentrations from 0.1 nM to 1 \(\mu\)M (Fig. 1, A and B). When ouabain concentration is increased further, a greater elevation of [Ca\(^{2+}\)]\(_{\text{CYT}}\) is observed (Fig. 1, C-E). We interpret those findings as evidence that both high-ouabain affinity (\(\alpha_2/\alpha_3\)) and low-affinity (\(\alpha_1\)) Na\(^{+}\) pump isoforms participate in the modulation of [Ca\(^{2+}\)]\(_{\text{CYT}}\).

The biphasic pattern of [Ca\(^{2+}\)]\(_{\text{CYT}}\) in Fig. 1 is atypical of pure NCX inhibition. That observation stimulated us to examine whether other pathways participate in acute ouabain [Ca\(^{2+}\)]\(_{\text{CYT}}\) elevation. Much work by Xie and colleagues (40, 50) highlighted the ability of ouabain binding to Na\(^{+}\) pumps to trigger multiple signaling cascades that occur along with NCX inhibition. Of particular interest in the current context is that ouabain may stimulate phosphorylation of PLC\(\gamma\) leading to InsP\(_3\) generation and biphasic [Ca\(^{2+}\)]\(_{\text{JNT}}\) signaling in LLC-PK\(_1\) cells (51). Cross talk between these signaling pathways has been proposed. NCX inhibition by Na\(^{+}\) elevation in the junctional region between plasmalemma and ER/SR ([Na\(^{+}\)]\(_{\text{JNT}}\)) might enhance InsP\(_3\)-mediated signaling if resultant junctional [Ca\(^{2+}\)]\(_{\text{JNT}}\) elevation provides positive feedback to further stimulate PLC (23). [Ca\(^{2+}\)]\(_{\text{JNT}}\) elevation might also induce CICR through InsP-R stimulation (Fig. 10). Our observations support the participation of multiple pathways in ouabain signaling. SEA-0400 prevented ouabain responses, implicating an important role for NCX1 isoforms (Fig. 5). A role for InsP-R stimulation is favored by the successful blockade of ouabain responses with 2-APB (Fig. 6). Finally, the ability of low [Ca\(^{2+}\)]\(_{\text{JNT}}\) (Fig. 3) as well as cation channel blockade with La\(^{3+}\) and SKF-96365 (Fig. 7) to attenuate ouabain responses points to a possible role for modulation of Ca\(^{2+}\) entry.
NCX modulates DVR endothelial [Ca\(^{2+}\)]\(_{\text{CYT}}\). Participation of NCX in the setting of basal [Ca\(^{2+}\)]\(_{\text{CYT}}\) of DVR endothelia has not been previously explored. In this study, a role for NCX was supported by two experiments. First, a progressive rise of endothelial [Ca\(^{2+}\)]\(_{\text{CYT}}\) occurs in response to step decreases of [Na\(^{+}\)]\(_o\), even when the [Na\(^{+}\)]\(_o\) reduction is small (Fig. 4). Second, NCX blockade with SEA-0400 raises [Ca\(^{2+}\)]\(_{\text{CYT}}\) elevation that did not persist (49). In other studies, progressive [Ca\(^{2+}\)]\(_{\text{CYT}}\) elevation was observed during [Na\(^{+}\)]\(_o\) reduction that mirrors our results (14, 26, 27). Such variation may reflect regional differences in the role of NCX at sites along the vasculature. Taken together, we propose that NCX may participate, along with plasma-luminal ATP-dependent Ca\(^{2+}\) extrusion pumps, to maintain DVR endothelial [Ca\(^{2+}\)]\(_{\text{CYT}}\) at low levels in the basal state.

Ouabain potentiates BK-induced calcium transients. In addition to inhibition of NCX, elevations of [Ca\(^{2+}\)]\(_{\text{CYT}}\) by ouabain probably involve release of Ca\(^{2+}\) from cellular stores. The tendency of ouabain and NCX inhibition (low [Na\(^{+}\)]\(_o\) or SEA-0400) to yield nonsustained “peak” [Ca\(^{2+}\)]\(_{\text{CYT}}\) transients hints at that possibility (Figs. 1, 4, 5A). The finding that 2-APB prevents both ouabain-induced [Ca\(^{2+}\)]\(_{\text{CYT}}\) transients (Fig. 6) and NCX-mediated transients (Fig. 8) is also supportive. Unfortunately, a perfect blocker of ER/SR store release via InsP\(_3\)R does not exist. Heparin, xestospongin C, and 2-APB are commonly used to block InsP\(_3\)R but neither is perfectly selective (10, 11). 2-APB, used in the present study, has dual actions in addition to inhibition of NCX, elevations of [Ca\(^{2+}\)]\(_{\text{CYT}}\) from cellular stores.

To further examine the role of cellular Ca\(^{2+}\) stores in ouabain responses, we tested its effects on BK signaling. BK is an agonist that releases Ca\(^{2+}\) from the ER/SR by InsP\(_3\)R-dependent signaling (4) and is an optimal agonist in the current context because it consistently generates rapid and large transient elevations of DVR endothelial [Ca\(^{2+}\)]\(_{\text{CYT}}\) (37, 41, 46). Augmentation of the peak phase of store release by ouabain was readily demonstrated at both high and low ouabain concentrations (Fig. 8). That observation parallels the recent finding that ouabain enhances [Ca\(^{2+}\)]\(_{\text{CYT}}\) transients in BK-stimulated rat aortic endothelial cells (14). A similar ability of ouabain to enhance [Ca\(^{2+}\)]\(_{\text{CYT}}\) transients in smooth muscle has also been described (1, 2).
A hypothesis that accounts for the effects of ouabain in DVR endothelium must explain its modulation of global $\text{[Ca}^{2+}]_{\text{CYT}}$ (as measured by fura-2) and its dependence on NCX, cation channels, and InsP$_3$R activity. A possible scheme is illustrated in Fig. 10. It has been proposed that ouabain-induced subplasmalemmal $\text{[Ca}^{2+}]_{\text{INT}}$ elevation, resulting from reduction of NCX $\text{Ca}^{2+}$ export, enhances $\text{Ca}^{2+}$ loading into ER/SR stores via SERCA pumps (17). In support of this, a steep dependence of agonist-induced, InsP$_3$-mediated $\text{Ca}^{2+}$ release on the ER/SR load of the stores has been reported in myocytes (45). Another mechanism, possibly contributing to ouabain-induced global $\text{[Ca}^{2+}]_{\text{CYT}}$ elevations, is CICR. It is known that $\text{Ca}^{2+}$ elevations associated with the reverse mode operation of NCX evoke CICR that amplifies $\text{Ca}^{2+}$ responses in cardiac myocytes (4, 5, 48). In pancreatic $\beta$-cells, CICR induced by SERCA pump inhibition was found to be dependent on InsP$_3$R (15). CICR is less well explored in endothelia but does exist (13, 31, 34). Comparison of the effect of low [Na]$^+$ on $\text{[Ca}^{2+}]_{\text{CYT}}$ in the presence or absence of 2-APB (Fig. 8) reveals that step decreases in [Na]$^+$, evoke $\text{[Ca}^{2+}]_{\text{CYT}}$ transients. These may be triggered by NCX inhibition through the CICR mechanism. In addition to possible InsP$_3$ generation (51), the same mechanism might partially account for the observed transient peaks of $\text{[Ca}^{2+}]_{\text{CYT}}$ elicited by ouabain (Fig. 1). Based on these observations, we conclude that circulating OLF might affect DVR endothelial $\text{Ca}^{2+}$ signaling through actions on NCX and InsP$_3$R. Within the renal medulla, the potential for ouabain to modulate release of vasodilators by DVR endothelia can be inferred. Given that vasoactivity and NO release in the renal medulla affect Na$^+$ balance and blood pressure (12), the current observations may point to an important role for circulating OLF in renal function.

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