Am J Physiol Renal Physiol 291: F761–F769, 2006.
First published April 4, 2006; doi:10.1152/ajprenal.00326.2005.

Ouabain modulation of endothelial calcium signaling in descending vasa recta

János Pittner,[1] Kristie Rhinehart,[1] and Thomas L. Pallone[1,2]

[1]*Division of Nephrology, Department of Medicine, and* [2]*Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland*

Submitted 12 August 2005; accepted in final form 27 March 2006

Pittner, János, Kristie Rhinehart, and Thomas L. Pallone. 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 ($[Ca^{2+}]_{CYT}$) in rat descending vasa recta (DVR). Over a broad range between 10^{-10} and 10^{-4} M, ouabain elicited biphasic peak and plateau $[Ca^{2+}]_{CYT}$ elevations. Blockade of voltage-gated Ca^{2+} entry with nifedipine did not affect the response to ouabain mitigating against a role for myo-endothelial gap junctions. Reduction of extracellular Na^+ concentration ($[Na^+]_o$) or Na^+/Ca^{2+} exchanger (NCX) inhibition with SEA-0400 (10^{-6} M) elevated $[Ca^{2+}]_{CYT}$, supporting a role for NCX in the setting of basal $[Ca^{2+}]_{CYT}$. SEA-0400 abolished the $[Ca^{2+}]_{CYT}$ response to ouabain implicating NCX as a mediator. The transient peak phase of $[Ca^{2+}]_{CYT}$ elevation that followed either ouabain or reduction of $[Na^+]_o$ was abolished by 2-aminoethoxydiphenyl borate (5×10^{-5} M). Cation channel blockade with La^{3+} (10 μM) or SKF-96365 (10 μM) also attenuated the ouabain-induced $[Ca^{2+}]_{CYT}$ response. Ouabain pretreatment increased the $[Ca^{2+}]_{CYT}$ elevation elicited by bradykinin (10^{-7} M). We conclude that inhibition of ouabain-sensitive Na^+-K^+-ATPase enhances DVR endothelial Ca^{2+} store loading and modulates $[Ca^{2+}]_{CYT}$ signaling through mechanisms that involve NCX, Ca^{2+} release, and cation channel activation.

kidney; medulla; fura 2; SEA-0400; bradykinin; 2-aminoethoxydiphenyl 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 Ca^{2+} export by Na^+/Ca^{2+} exchange (NCX). One important effect of the decrease in Ca^{2+} export may be to augment loading of Ca^{2+} into endoplasmic/sarcoplasmic reticulum (ER/SR) stores. Through that putative mechanism, ouabain has been shown to augment agonist-induced cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{CYT}$) 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 ($InsP_3$) generation. In contrast to effects on smooth muscle and cardiac myocytes, the role of ouabain to modulate microvascular endothelial $[Ca^{2+}]_{CYT}$ has not been as thoroughly explored.

Descending vasa recta (DVR) are 15-μm-diameter branches of juxtamedullary efferent 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 $[Ca^{2+}]_{CYT}$ to release vasodilators and limit DVR vasoconstriction (12, 37, 38, 43). Freshly isolated DVR are an attractive model to study endothelial $[Ca^{2+}]_{CYT}$ responses in an intact microvessel preparation because the Ca^{2+}-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 $[Ca^{2+}]_{CYT}$. Our results verify that ouabain, over a broad range of concentrations, increases basal $[Ca^{2+}]_{CYT}$ in a biphasic manner. Na^+/Ca^{2+} exchanger (NCX) and other pathways participate in the response. NCX blockade with SEA-0400, inositol trisphosphate receptor ($InsP_3R$) blockade with 2-aminoethoxydiphenyl borate (2-APB), and nonselective cation channel blockade with La^{3+} or SKF-96365 interfere with the actions of ouabain. Finally, prolonged ouabain pretreatment increased the magnitude of $[Ca^{2+}]_{CYT}$ elevation induced by BK suggesting enhancement of store loading of Ca^{2+} 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 $MgSO_4$, 1.2 Na_2HPO_4, 5 HEPES, 5 D-glucose, 5 L-alanine, 0.1 L-arginine, and 1 $CaCl_2$. 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 $[Ca^{2+}]_{CYT}$. DVR were loaded with fura 2-AM (5 μM) added to the bath at 37°C for 15 min. We

Address for reprint requests and other correspondence: T. L. Pallone, Division of Nephrology, UMMS, 22 S. Greene St, N3W143, Univ. of Maryland at Baltimore, Baltimore, MD 21201-1595 (e-mail: tpallone@medicine.umaryland.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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 $[Ca^{2+}]_{CYT}$ 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 510WB40 (Omega optical) filter. Background-subtracted fluorescence emission ratios ($R_{350/380}$) were converted to $[Ca^{2+}]_{CYT}$ assuming a dissociation constant for fura 2 of 224 nM. R_{min} and R_{max} were measured in vessels exposed to 10^{-5} M ionomycin with 0 $CaCl_2$ and 5×10^{-4} M EGTA, or 5×10^{-3} M $CaCl_2$, 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 kaspartate, 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 $MgCl_2$, 1 $CaCl_2$, 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.

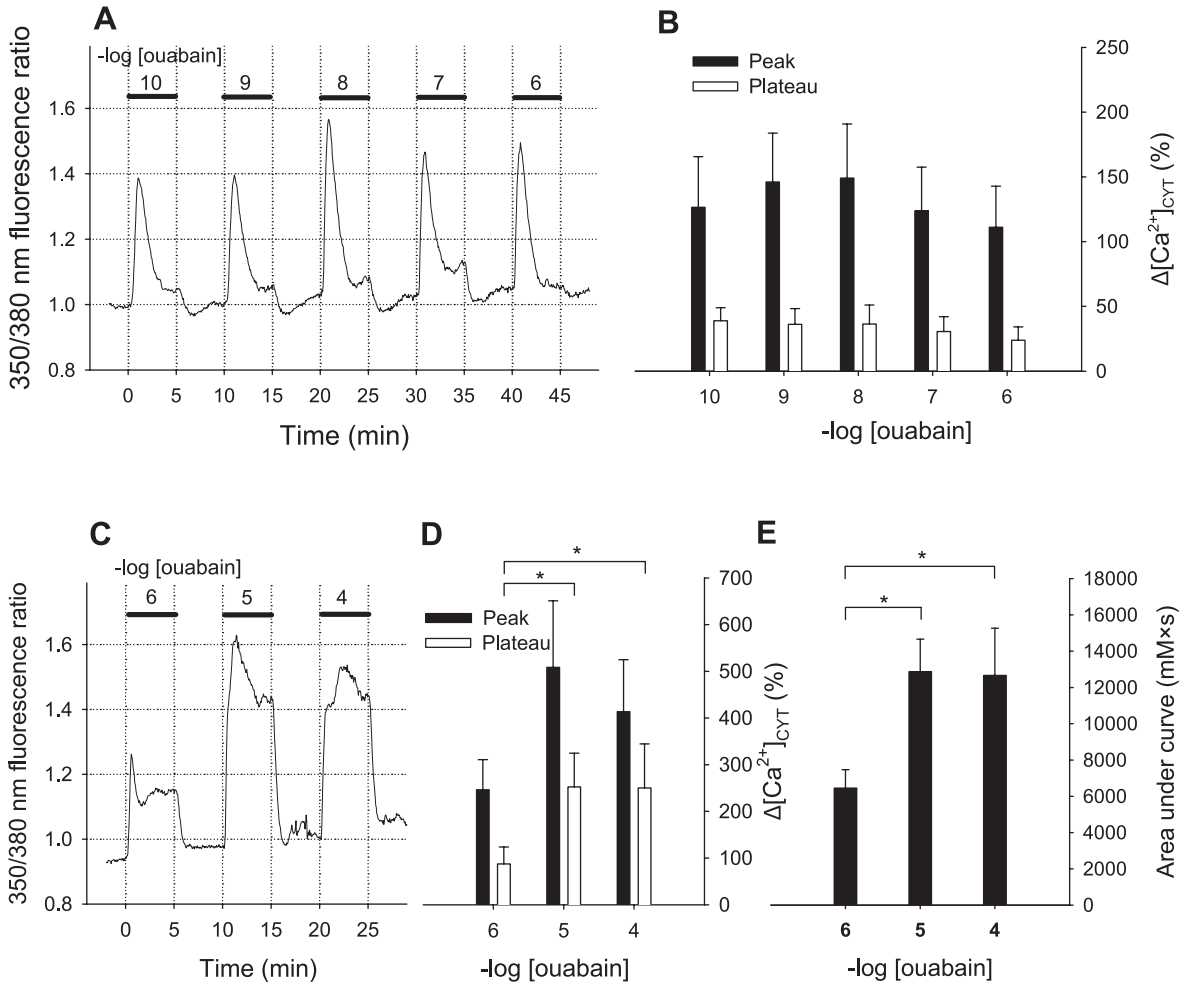

Fig. 1. Descending vasa recta (DVR) endothelial cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{CYT}$) changes evoked by ouabain. Vessels were exposed to log molar increasing concentrations of ouabain from 10^{-10} to 10^{-6} M ($n = 9$). At each concentration, ouabain was introduced for 5 min and then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

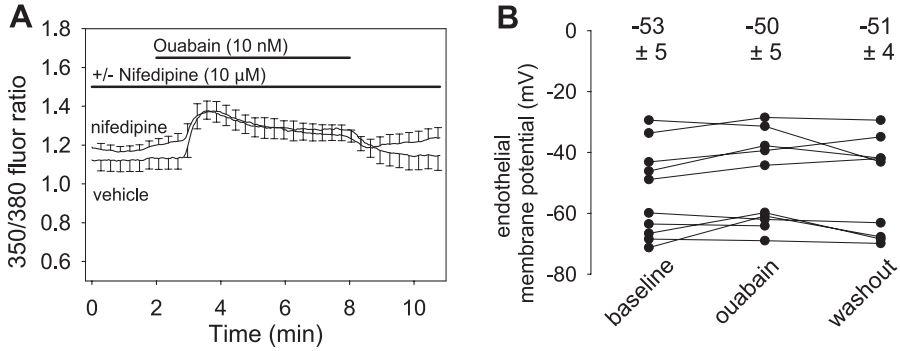

Fig. 2. Effect of inhibition of voltage-operated Ca^{2+} channels (VOCa) on ouabain-evoked DVR endothelial $[Ca^{2+}]_{CYT}$ transients. Ouabain was added 5 min after the introduction of nifedipine (10^{-5} M) or sham exchange of the bath. *A*: mean background-subtracted fluorescent ratios are shown as a function of time during ouabain (10 nM) exposure in the presence or absence of nifedipine (10 μM). *B*: membrane potential of DVR endothelial cells before, during, and after exposure to ouabain (10 nM).

RESULTS

Modulation of DVR endothelial $[Ca^{2+}]_{CYT}$ by ouabain. We first tested whether inhibition of ouabain-sensitive Na^+-K^+-ATPase affects basal endothelial $[Ca^{2+}]_{CYT}$. Baseline DVR endothelial $[Ca^{2+}]_{CYT}$ 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 μM led to increases in $[Ca^{2+}]_{CYT}$ characterized by a transient peak and sustained plateau. $[Ca^{2+}]_{CYT}$ changes were reversible when ouabain was removed from the bath. The magnitudes of the increases in peak and plateau phases of the response were similar from 10^{-10} to 10^{-6} M ouabain (Fig. 1, *A* and *B*). Sham exchange of the bath with vehicle did not elicit such responses.

The ouabain affinity of α_2/α_3 isoforms of Na^+-K^+-ATPase in the rat has been reported to lie near or below 1 nM. In contrast, the more predominant α_1 isoform has a lower affinity, near 100 μM (6, 16, 33). To test whether higher ouabain concentrations that inhibit all Na^+-K^+-ATPase isoforms have a more pronounced effect on $[Ca^{2+}]_{CYT}$, a separate series of experiments was performed comparing ouabain at 10^{-6}-10^{-4} M. As shown in Fig. 1, *C-E*, a larger $[Ca^{2+}]_{CYT}$ elevation occurred at 10^{-4} and 10^{-5} than at 10^{-6} M (plateau phase; 75.3 ± 8.2 vs. 155.9 ± 24.3 and 149.3 ± 32.9 nM, area under curve; 6,447 ± 1,021 vs. 12,864 ± 1,801 and 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 $[Ca^{2+}]_{CYT}$ 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 $[Ca^{2+}]_{CYT}$ 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^{-5} M) and then exposed to ouabain (10 nM). Nifedipine did not inhibit ouabain-evoked $[Ca^{2+}]_{CYT}$ transients (Fig. 2*A*).

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. 2*B*). 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 $[Ca^{2+}]_{CYT}$ transients.

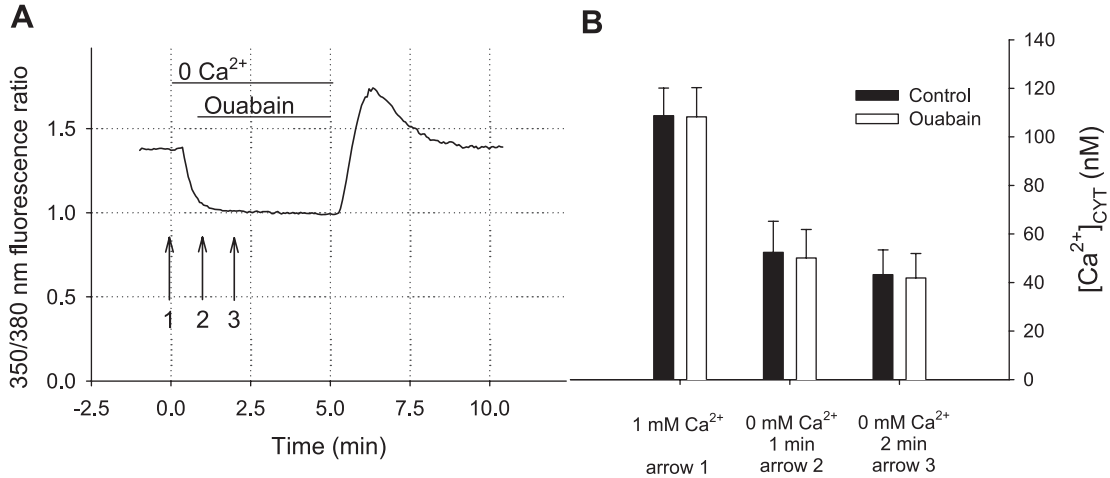

Fig. 3. Effect of removal of Ca^{2+} from the bath on ouabain-evoked DVR endothelial $[Ca^{2+}]_{CYT}$ transients. Ouabain was introduced or sham exchange was performed 1 min after elimination of Ca^{2+} from the bath. *A*: representative recording of a single experiment shows background-subtracted fluorescent ratios. Arrows indicate time points corresponding to comparisons in *B*. *B*: bars show $[Ca^{2+}]_{CYT}$ 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.

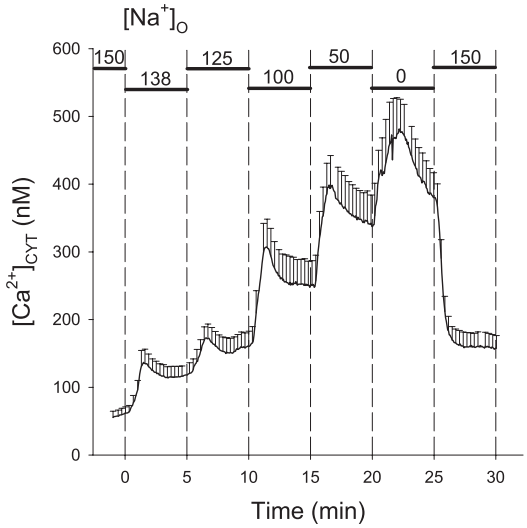

Fig. 4. Effect of lowering of $[Na^+]_o$ on DVR endothelial $[Ca^{2+}]_{CYT}$. $[Na^+]_o$ was sequentially lowered, at 5-min intervals, from 150 to 138, 125, 100, 50, and 0 mM by isosmolar substitution with $NMDG^+$, and finally restored to 150 mM ($n = 6$), $[Na^+]_o$ reductions yielded initial "peaks," followed by higher steady-state "plateau" levels of $[Ca^{2+}]_{CYT}$. 350/380 nm fura-2 fluorescent ratios were obtained at 0.2 Hz by averaging for 2.5 s at each wavelength. Data are means ± SE. Most error bars have been suppressed for clarity.

Role of NCX in the ouabain-induced endotheliol calcium transients. We hypothesized that, as in other cell types, ouabain inhibition of the α_2-, α_3-subunit sodium pumps might elevate $[Na^+]$ in the vicinity of the NCX thereby reducing clearance of Ca^{2+} from the endothelium. We first tested whether NCX activity can modulate $[Ca^{2+}]_{CYT}$ of DVR endothelia by lowering extracellular sodium ($[Na^+]_o$) or calcium concentration ($[Ca^{2+}]_o$). As shown in Fig. 3, removal of Ca^{2+} from the bath rapidly reduced $[Ca^{2+}]_{CYT}$ and eliminated ouabain (10^{-8} M)-induced $[Ca^{2+}]_{CYT}$ transients. Given that other effects such as ER/SR store depletion and alteration of Ca^{2+} export via Ca^{2+}-ATPase might accompany incubation of cells in 0 Ca^{2+} bath, we also tested the effect of lowering $[Na^+]_o$. Stepwise reduction of $[Na^+]_o$ elicited incremental elevations of $[Ca^{2+}]_{CYT}$ (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 $[Ca^{2+}]_{CYT}$ transients with peaks followed by persistent plateau elevations. These data favor a role for participation of NCX in the setting of basal $[Ca^{2+}]_{CYT}$.

To further establish a role for NCX, we examined baseline $[Ca^{2+}]_{CYT}$ and ouabain-induced $[Ca^{2+}]_{CYT}$ 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 $[Ca^{2+}]_{CYT}$ (control, 71.7 ± 5.1 nM, vs. SEA-0400, 179 ± 40.9 nM, $n = 6$ each, after 5 min at *arrow 1*, Fig. 5*A*). Furthermore, 5-min pretreatment with SEA-0400 almost completely abolished ouabain-induced $[Ca^{2+}]_{CYT}$ 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. 5*B*).

2-APB and cation channel blockade inhibits ouabain-evoked calcium transients. Hypothetically, inhibition of NCX could explain the ability of ouabain to elevate $[Ca^{2+}]_{CYT}$ without invoking a need for participation of other Ca^{2+} transport pathways. The biphasic "peak and plateau" $[Ca^{2+}]_{CYT}$ responses shown in Figs. 1–5, however, raise the question of release of Ca^{2+} from internal stores and influx of Ca^{2+} from the extracellular space via store-operated nonselective cation channels. Given that ouabain enhances storage of Ca^{2+} in some cells (18) and has recently been shown to signal through PLC and $InsP_3$ in renal epithelial ($LLC\text{-}PK_1$) cells (50, 51), we tested other pathways. A selective blocker of $InsP_3R$-mediated Ca^{2+} release from stores does not exist; however, 2-APB (5×10^{-5} M) blocks $InsP_3R$ along with store-operated Ca^{2+} channels (10, 11). As shown in Fig. 6, 2-APB decreased baseline endothelial $[Ca^{2+}]_{CYT}$ (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 $[Ca^{2+}]_{CYT}$ 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 $InsP_3$ generation occurs in DVR endothelia exposed to ouabain, secondary activation of Ca^{2+} influx via nonselective 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 La^{3+} (10 μM), agents that are known to block mechanosensi-

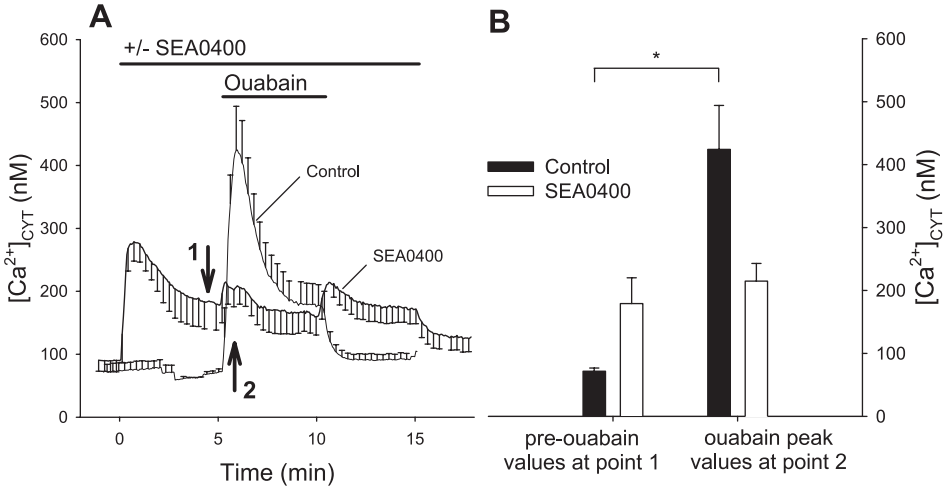

Fig. 5. Effect of SEA-0400 pretreatment on ouabain-induced $[Ca^{2+}]_{CYT}$ transients in DVR endothelium. *A*: SEA-0400 or vehicle was added to the bath 5 min before introduction of ouabain. Na^+/Ca^{2+} exchanger (NCX) inhibition with SEA-0400 elevated baseline $[Ca^{2+}]_{CYT}$. Ouabain exposure was continued for 5 min. $[Ca^{2+}]_{CYT}$ responses to ouabain following SEA-0400 pretreatment were suppressed. *B*: peak $[Ca^{2+}]_{CYT}$ changes induced by ouabain are compared. Data are means ± SE, $*P < 0.05$.

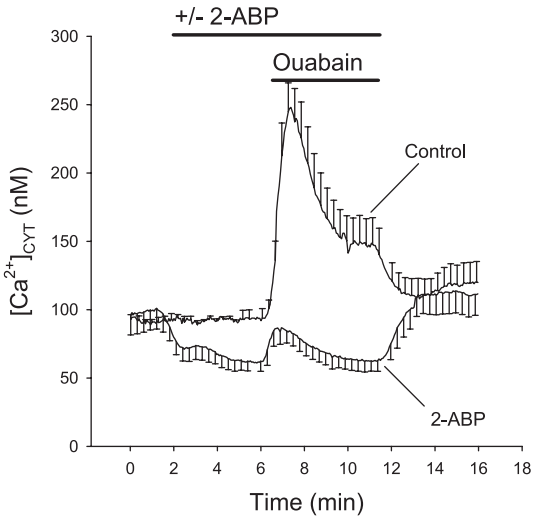

Fig. 6. Effect of 2-aminoethoxydiphenyl borate (2-APB) on ouabain-induced $[Ca^{2+}]_{CYT}$ transients. Fura-2-loaded DVR were pretreated with 2-APB (5×10^{-5} M) or vehicle before ouabain exposure. Baseline $[Ca^{2+}]_{CYT}$ was lower in 2-APB and ouabain-evoked peak $[Ca^{2+}]_{CYT}$ elevations were suppressed. Data are means ± SE.

tive $[Ca^{2+}]_{CYT}$ transients in these cells (53). La^{3+} ion, at a concentration of 10 μM, spares inhibition of NCX and Ca^{2+} extrusion pumps (9, 44). As shown in Fig. 7, both agents attenuated the $[Ca^{2+}]_{CYT}$ response to ouabain.

As with the response to ouabain (Fig. 1), transient peak $[Ca^{2+}]_{CYT}$ elevation accompanies inhibition of NCX through $[Na^+]_o$ reduction (Fig. 4). It has been hypothesized that localized $[Ca^{2+}]_{CYT}$ elevations near NCX might stimulate store Ca^{2+} release through stimulation of PLC generation of $InsP_3$ or by direct actions on $InsP_3R$ (Ca^{2+}-induced Ca^{2+} release). In view of this, we tested the ability of 2-APB to block the $[Ca^{2+}]_{CYT}$ response to $[Na^+]_o$ reduction. The protocol used in Fig. 4 was repeated, but with 2-APB present in the bath (Fig. 8). As in Fig. 6, 2-APB lowered baseline $[Ca^{2+}]_{CYT}$. The transients (arrows, Fig. 8) associated with putative store release were suppressed (control, $n = 6$ vs. 2-APB, $n = 8$; 150 mM $[Na^+]_o$: 61.7 ± 9.5 vs. 41.5 ± 4.2, 138 mM: 118 ± 15.3 vs. 85.2 ± 13.3, 125 mM: 160 ± 22.6 vs. 124 ± 19.2, 100 mM: 250 ± 36.7 vs. 208 ± 36.0, 50 mM: 342 ± 41.5 vs. 331 ± 47, 0 mM: 380 ± 37.0 vs. 381 ± 83.8 nM). Attenuation of the $[Ca^{2+}]_{CYT}$ response by 2-APB implies participation of Ca^{2+} release or entry pathways but the persistence of the stepwise increase in plateau $[Ca^{2+}]_{CYT}$ in 2-APB suggests that it does not inhibit NCX.

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 μM). 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. 8*A*).

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 (5×10^{-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 × 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^{-8} M) selective for α_2/α_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 × s, $n = 7$, $P < 0.05$, Fig. 9, *C* and *D*).

DISCUSSION

Four isoforms of the Na^+-K^+-ATPase catalytic subunit (α_1-α_4) are expressed in mammalian cells. The α_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 α_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 α_2-, α_3-, or α_4-isoforms (22, 30). Reduction of Na^+ export then leads to an increase in localized $[Na^+]_{CYT}$ (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 α_2/α_3-isoforms may be localized to discrete func-

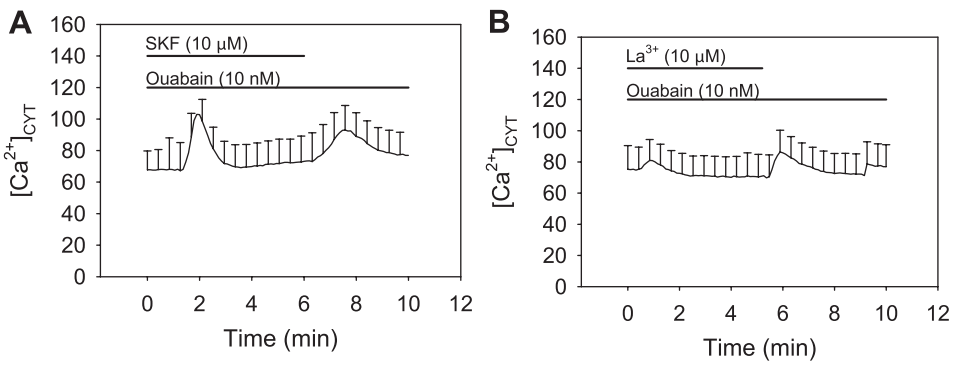

Fig. 7. Effect of cation channel blockade on ouabain-induced $[Ca^{2+}]_{CYT}$ transients. Fura-2-loaded DVR endothelia were exposed to ouabain (10 nM) in the presence of nonselective cation channel blockade with SKF-96365 (10 μM; *A*) or La^{3+} (10 μM; *B*). Both agents attenuated the $[Ca^{2+}]_{CYT}$ response to ouabain (compare with Figs. 1, 5, 6).

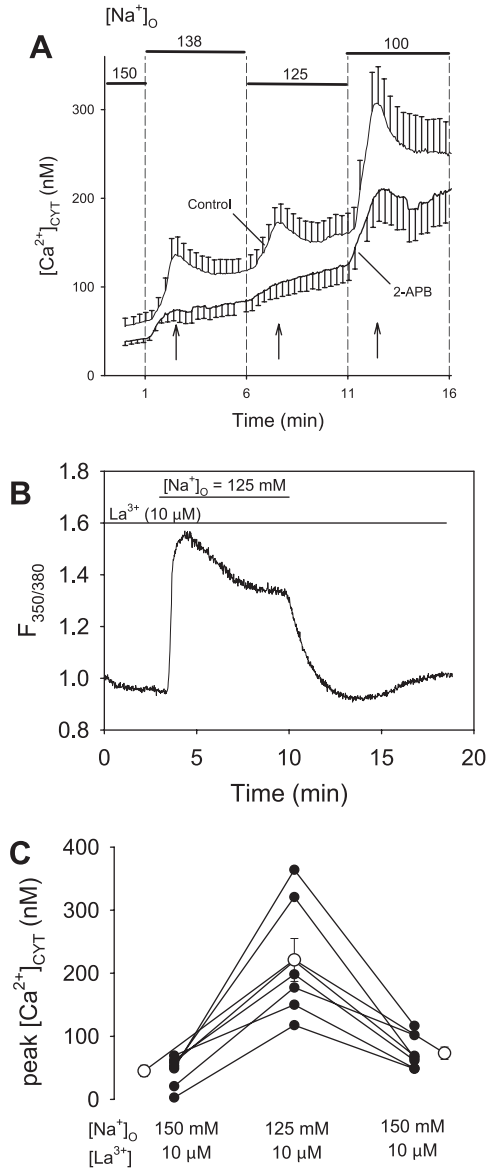

Fig. 8. Effect of 2-APB and La^{3+} on DVR endothelial $[Ca^{2+}]_{CYT}$ response to $[Na^+]_o$ reduction. *A*: protocol illustrated in Fig. 4 was repeated in the presence of 2-APB (5×10^{-5} M). $[Na^+]_o$ reduction induced a progressive elevation of $[Ca^{2+}]_{CYT}$ in the presence or absence of 2-APB. Baseline $[Ca^{2+}]_{CYT}$ was lower in 2-APB than vehicle and $[Ca^{2+}]_{CYT}$ peaks, indicated by arrows, were diminished. *B*: example of an experiment in which the ability of nonselective cation channel blockade with La^{3+} (10 μM) to prevent $[Ca^{2+}]_{CYT}$ responses to $[Na^+]_o$ reduction was tested. *C*: summary of $n = 7$ experiments similar to *B*. ●, Individual experiments. ○, Means ± SE.

tional units within cells. Recent investigations in astrocytes and mesenteric arteriolar myocytes found the ouabain-sensitive α_2/α_3-isoforms and NCX in specialized "junctional" regions that coincide with ER/SR (6, 8, 16, 18, 25). Similar colocalization of α_2/α_3 and NCX in DVR endothelium is unexplored; however, the ability of $[Na^+]_o$ reduction to increase $[Ca^{2+}]_{CYT}$ in DVR endothelia (Fig. 4) favors a role for NCX in the setting of basal $[Ca^{2+}]_{CYT}$ and reproduces the findings of others (27). A role for NCX is also favored by the ability of SEA-0400 to increase basal $[Ca^{2+}]_{CYT}$ (Fig. 5). SEA-0400 is a potent blocker of predominant NCX1 isoforms and is generally respected for its specificity to block NCX without affecting cytoplasmic Ca^{2+} extrusion pumps (24, 29, 47). Nonetheless, some interpretational caution is in order because SEA-0400 has been shown to alter $[Ca^{2+}]_{CYT}$ 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 $[Ca^{2+}]_{CYT}$ (Fig. 1). The lower end of that concentration range is similar to that of OLF in plasma (20) and is sufficient to affect α_2/α_3 Na^+-K^+-ATPase in the rat. Several findings in Fig. 1 are of interest. First, ouabain modulates basal $[Ca^{2+}]_{CYT}$ 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 $[Ca^{2+}]_{CYT}$ elevation occurs globally throughout the cells. It is possible that much greater effects on $[Ca^{2+}]_{CYT}$ 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+} ($[Ca^{2+}]_{JNT}$) 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 $[Ca^{2+}]_{CYT}$ change comprised of an early peak followed by a sustained plateau. Observation times in Fig. 1 were brief; however, plateau $[Ca^{2+}]_{CYT}$ 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 μM (Fig. 1, *A* and *B*). When ouabain concentration is increased further, a greater elevation of $[Ca^{2+}]_{CYT}$ is observed (Fig. 1, *C*-*E*). We interpret those findings as evidence that both high-ouabain affinity (α_2/α_3) and low-affinity (α_1) Na^+ pump isoforms participate in the modulation of $[Ca^{2+}]_{CYT}$.

The biphasic pattern of $[Ca^{2+}]_{CYT}$ in Fig. 1 is atypical of pure NCX inhibition. That observation stimulated us to examine whether other pathways participate in acute ouabain $[Ca^{2+}]_{CYT}$ 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 PLCγ leading to $InsP_3$ generation and biphasic $[Ca^{2+}]_{CYT}$ 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 ($[Na^+]_{JNT}$) might enhance $InsP_3$-mediated signaling if resultant junctional $[Ca^{2+}]_{JNT}$ elevation provides positive feedback to further stimulate PLC (23). $[Ca^{2+}]_{JNT}$ elevation might also induce CICR through $InsP_3R$ 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 $InsP_3R$ stimulation is favored by the successful blockade of ouabain responses with 2-APB (Fig. 6). Finally, the ability of low $[Ca^{2+}]_o$ (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.

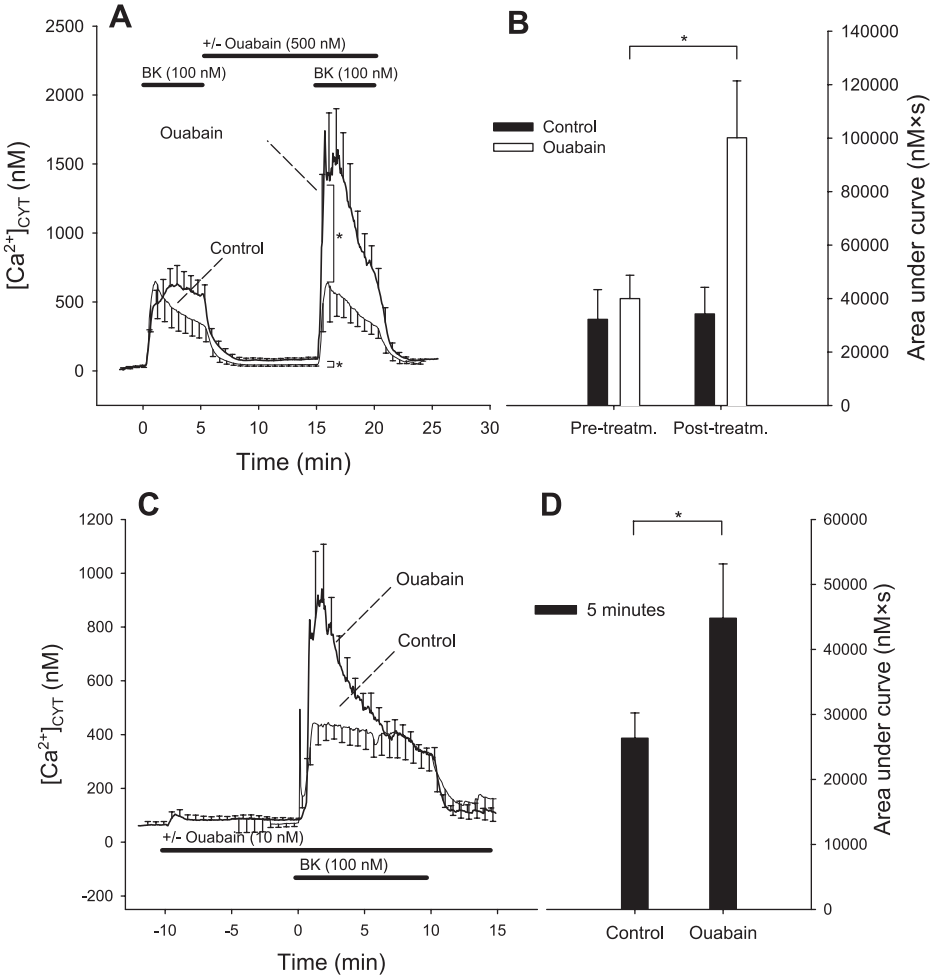

Fig. 9. Effect of ouabain on DVR endothelial $[Ca^{2+}]_{CYT}$ elevation evoked by bradykinin (BK). *A*: DVR were exposed to BK for 5 min to elicit a baseline response. Subsquently, vehicle (n = 6) or ouabain (500 nM, n = 9) was introduced into the bath for 5 min and vessels were exposed to BK a second time. Vessels exposed to ouabain had significant elevation of $[Ca^{2+}]_{CYT}$ (*$P < 0.05$). The second BK-induced $[Ca^{2+}]_{CYT}$ response was higher after ouabain (*$P < 0.05$). *B*: summary of area under the curve comparisons of $[Ca^{2+}]_{CYT}$ during the first and second exposures to BK (mean ± SE, *$P < 0.05$). Ouabain enhanced the peak phase BK-induced $[Ca^{2+}]_{CYT}$ response. *C*: DVR were pre-exposed to ouabain (10 nM) or vehicle for 10 min followed by 10-min BK stimulation (10^{-7} M). *D*: area under the curve comparison for the first 5 min of BK exposure in *C*. At lower concentration, ouabain (10 nM) also enhanced the peak phase $[Ca^{2+}]_{CYT}$ response. Data are means ± SE.

NCX modulates DVR endothelial $[Ca^{2+}]_{CYT}$. Participation of NCX in the setting of basal $[Ca^{2+}]_{CYT}$ 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 $[Ca^{2+}]_{CYT}$ 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 $[Ca^{2+}]_{CYT}$ (Fig. 5). In one study, conducted in aortic endothelial cells, lowering $[Na^+]_o$ caused a transient $[Ca^{2+}]_{CYT}$ elevation that did not persist (49). In other studies, progressive $[Ca^{2+}]_{CYT}$ 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 plasmalemmal ATP-dependent Ca^{2+} extrusion pumps, to maintain DVR endothelial $[Ca^{2+}]_{CYT}$ at low levels in the basal state.

Ouabain potentiates BK-induced calcium transients. In addition to inhibition of NCX, elevations of $[Ca^{2+}]_{CYT}$ 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" $[Ca^{2+}]_{CYT}$ transients hints at that possibility (Figs. 1, 4, 5*A*). The finding that 2-APB prevents both ouabain-induced $[Ca^{2+}]_{CYT}$ transients (Fig. 6) and NCX-mediated transients (Fig. 8) is also supportive. Unfortunately, a perfect blocker of ER/SR store release via $InsP_3R$ does not exist. Heparin, xestospongin C, and 2-APB are commonly used to block $InsP_3R$ but neither is perfectly selective (10, 11). 2-APB, used in the present study, has dual actions to inhibit $InsP_3R$ and block Ca^{2+} entry from the extracellular space through store-operated cation channels. Participation of Ca^{2+} entry in the ouabain response is supported by the finding that La^{3+} and SKF-96365 attenuate it. Given the imperfect specificity of 2-APB, we recognize that evidence for participation of $InsP_3R$ participation in the ouabain response is incomplete. Nonetheless, the clear demonstration by Yuan and colleagues (51) that $InsP_3$ generation can be stimulated through ouabain binding to Na^+ pump α-subunits reinforces the possibility.

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 $InsP_3R$-dependent signaling (4) and is an optimal agonist in the current context because it consistently generates rapid and large transient elevations of DVR endothelial $[Ca^{2+}]_{CYT}$ (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 $[Ca^{2+}]_{CYT}$ transients in BK-stimulated rat aortic endothelial cells (14). A similar ability of ouabain to enhance $[Ca^{2+}]_{CYT}$ transients in smooth muscle has also been described (1, 2).

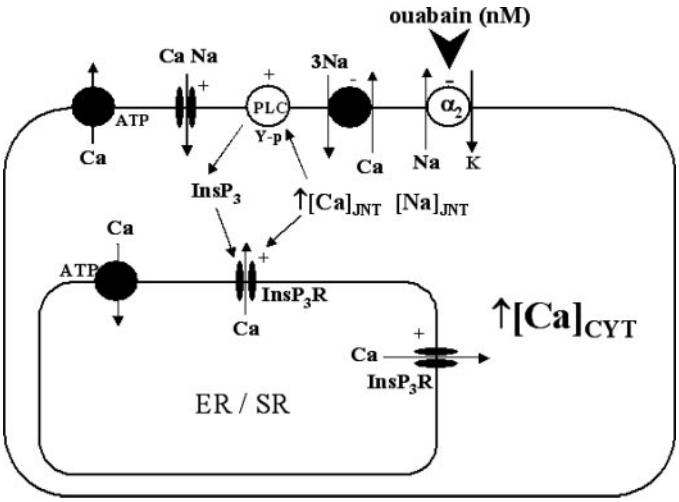

Fig. 10. Mechanisms of ouabain-induced $[Ca^{2+}]_{CYT}$ signaling in DVR endothelium. Possible scheme involving participation of NCX, inositol tris-phosphate receptor ($InsP_3R$), and cation channels in DVR endothelial ouabain signaling. Nanomolar ouabain selectively inhibits those Na^+-K^+-ATPase comprised of α_2-catalytic subunits. That inhibition raises $[Na^+]_{JNT}$ in the vicinity of NCX of cytoplasmic "junctional" microdomains that overlie endoplasmic/sarcoplasmic reticulum (ER/SR) Ca^{2+} stores (2, 6). Inhibition of Ca^{2+} export by NCX leads to increase in junctional $[Ca^{2+}]_{JNT}$ favoring enhanced uptake into ER/SR stores via Ca^{2+} pumps. The greater load of Ca^{2+} in ER/SR stores favors higher "bulk" $[Ca^{2+}]_{CYT}$ and greater agonist-induced Ca^{2+} release via $InsP_3R$. Parallel signaling processes occur through stimulation of PLC, which may be phosphorylated (Y-p) (51) and stimulated by the increase in $[Ca^{2+}]_{JNT}$ (23). $InsP_3$ generated by PLC combined with the increase in $[Ca^{2+}]_{JNT}$ due to NCX inhibition stimulates further Ca^{2+} release by $InsP_3R$ leading to secondary opening of store-operated nonselective cation channels that transport Na^+ and Ca^{2+} ions.

A hypothesis that accounts for the effects of ouabain in DVR endothelium must explain its modulation of global $[Ca^{2+}]_{CYT}$ (as measured by fura-2) and its dependence on NCX, cation channels, and $InsP_3R$ activity. A possible scheme is illustrated in Fig. 10. It has been proposed that ouabain-induced subplasmalemmal $[Ca^{2+}]_{JNT}$ elevation, resulting from reduction of NCX Ca^{2+} export, enhances Ca^{2+} loading into ER/SR stores via SERCA pumps (17). In support of this, a steep dependence of agonist-induced, $InsP_3$-mediated Ca^{2+} release on the Ca^{2+} load of the stores has been reported in myocytes (45). Another mechanism, possibly contributing to ouabain-induced global $[Ca^{2+}]_{CYT}$ elevations, is CICR. It is known that Ca^{2+} elevations associated with the reverse mode operation of NCX evoke CICR that amplifies $[Ca^{2+}]_{CYT}$ responses in cardiac myocytes (4, 5, 48). In pancreatic β-cells, CICR induced by SERCA pump inhibition was found to be dependent on $InsP_3R$ (15). CICR is less well explored in endothelia but does exist (13, 31, 34). Comparison of the effect of low $[Na^+]_o$ on $[Ca^{2+}]_{CYT}$ in the presence or absence of 2-APB (Fig. 8) reveals that step decreases in $[Na^+]_o$ evoke $[Ca^{2+}]_{CYT}$ 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 $[Ca^{2+}]_{CYT}$ elicited by ouabain (Fig. 1). Based on these observations, we conclude that circulating OLF might affect DVR endothelial Ca^{2+} signaling through actions on NCX and $InsP_3R$. 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.

GRANTS

This work was supported by National Institutes of Health Grants DK-42495, DK-68492, DK-67621, and HL-78870.

REFERENCES

1. **Arnon A, Hamlyn JM, and Blaustein MP.** Na^+ entry via store-operated channels modulates Ca^{2+} signaling in arterial myocytes. *Am J Physiol Cell Physiol* 278: C163–C173, 2000.
2. **Arnon A, Hamlyn JM, and Blaustein MP.** Ouabain augments Ca^{2+} transients in arterial smooth muscle without raising cytosolic Na^+. *Am J Physiol Heart Circ Physiol* 279: H679–H691, 2000.
3. **Batlle DC, Godinich M, LaPointe MS, Munoz E, Carone F, and Mehring N.** Extracellular Na^+ dependency of free cytosolic Ca^{2+} regulation in aortic vascular smooth muscle cells. *Am J Physiol Cell Physiol* 261: C845–C856, 1991.
4. **Berridge MJ, Lipp P, and Bootman MD.** The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 1: 11–21, 2000.
5. **Bers DM.** Cardiac excitation-contraction coupling. *Nature* 415: 198–205, 2002.
6. **Blaustein MP and Golovina VA.** Structural complexity and functional diversity of endoplasmic reticulum Ca^{2+} stores. *Trends Neurosci* 24: 602–608, 2001.
7. **Blaustein MP, Juhaszova M, and Golovina VA.** The cellular mechanism of action of cardiotonic steroids: a new hypothesis. *Clin Exp Hypertens* 20: 691–703, 1998.
8. **Blaustein MP, Juhaszova M, Golovina VA, Church PJ, and Stanley EF.** Na/Ca exchanger and PMCA localization in neurons and astrocytes: functional implications. *Ann NY Acad Sci* 976: 356–366, 2002.
9. **Blaustein MP and Lederer WJ.** Sodium/calcium exchange: its physiological implications. *Physiol Rev* 79: 763–854, 1999.
10. **Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ, and Peppiatt CM.** 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of InsP3-induced Ca^{2+} release. *FASEB J* 16: 1145–1150, 2002.
11. **Castonguay A and Robitaille R.** Xestospongin C is a potent inhibitor of SERCA at a vertebrate synapse. *Cell Calcium* 32: 39–47, 2002.
12. **Cowley AW Jr, Mori T, Mattson D, and Zou AP.** Role of renal NO production in the regulation of medullary blood flow. *Am J Physiol Regul Integr Comp Physiol* 284: R1355–R1369, 2003.
13. **Domenighetti AA, Beny JL, Chabaud F, and Frieden M.** An intercellular regenerative calcium wave in porcine coronary artery endothelial cells in primary culture. *J Physiol* 513: 103–116, 1998.
14. **Dong XH, Komiyama Y, Nishimura N, Masuda M, and Takahashi H.** Nanomolar level of ouabain increases intracellular calcium to produce nitric oxide in rat aortic endothelial cells. *Clin Exp Pharmacol Physiol* 31: 276–283, 2004.
15. **Dyachok O, Tufveson G, and Gylfe E.** Ca^{2+}-induced Ca^{2+} release by activation of inositol 1,4,5-trisphosphate receptors in primary pancreatic beta-cells. *Cell Calcium* 36: 1–9, 2004.
16. **Golovina V, Song H, James P, Lingrel J, and Blaustein M.** Regulation of Ca^{2+} signaling by Na^+ pump alpha-2 subunit expression. *Ann NY Acad Sci* 986: 509–513, 2003.
17. **Golovina VA and Blaustein MP.** Unloading and refilling of two classes of spatially resolved endoplasmic reticulum Ca^{2+} stores in astrocytes. *Glia* 31: 15–28, 2000.
18. **Golovina VA, Song H, James PF, Lingrel JB, and Blaustein MP.** Na^+ pump α_2-subunit expression modulates Ca^{2+} signaling. *Am J Physiol Cell Physiol* 284: C475–C486, 2003.
19. **Hamlyn JM.** Biosynthesis of endogenous cardiac glycosides by mammalian adrenocortical cells: three steps forward. *Clin Chem* 50: 469–470, 2004.
20. **Hamlyn JM, Blaustein MP, Bova S, DuCharme DW, Harris DW, Mandel F, Mathews WR, and Ludens JH.** Identification and characterization of a ouabain-like compound from human plasma. *Proc Natl Acad Sci USA* 88: 6259–6263, 1991.
21. **Hamlyn JM, Lu ZR, Manunta P, Ludens JH, Kimura K, Shah JR, Laredo J, Hamilton JP, Hamilton MJ, and Hamilton BP.** Observations on the nature, biosynthesis, secretion and significance of endogenous ouabain. *Clin Exp Hypertens* 20: 523–533, 1998.

22. **Hansen O.** No evidence for a role in signal transduction of Na^+-K^+-ATPase interaction with putative endogenous ouabain. *Eur J Biochem* 270: 1916–1919, 2003.
23. **Horowitz LF, Hirdes W, Suh BC, Hilgemann DW, Mackie K, and Hille B.** Phospholipase C in living cells: activation, inhibition, Ca^{2+} requirement, and regulation of M current. *J Gen Physiol* 126: 243–262, 2005.
24. **Iwamoto T.** Forefront of Na^+/Ca^{2+} exchanger studies: molecular pharmacology of Na^+/Ca^{2+} exchange inhibitors. *J Pharm Sci* 96: 27–32, 2004.
25. **Juhaszova M and Blaustein MP.** Na^+ pump low and high ouabain affinity alpha subunit isoforms are differently distributed in cells. *Proc Natl Acad Sci USA* 94: 1800–1805, 1997.
26. **Kaye DM and Kelly RA.** Expression and regulation of the sodium-calcium exchanger in cardiac microvascular endothelial cells. *Clin Exp Pharmacol Physiol* 26: 651–655, 1999.
27. **Li L and van Breemen C.** Na^+-Ca^{2+} exchange in intact endothelium of rabbit cardiac valve. *Circ Res* 76: 396–404, 1995.
28. **Liang W, Buluc M, van Breemen C, and Wang X.** Vectorial Ca^{2+} release via ryanodine receptors contributes to Ca^{2+} extrusion from freshly isolated rabbit aortic endothelial cells. *Cell Calcium* 36: 431–443, 2004.
29. **Matsuda T, Arakawa N, Takuma K, Kishida Y, Kawasaki Y, Sakaue M, Takahashi K, Takahashi T, Suzuki T, Ota T, Hamano-Takahashi A, Onishi M, Tanaka Y, Kameo K, and Baba A.** SEA0400, a novel and selective inhibitor of the Na^+-Ca^{2+} exchanger, attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. *J Pharmacol Exp Ther* 298: 249–256, 2001.
30. **Mobasheri A, Errington RJ, Golding S, Hall AC, and Urban JP.** Characterization of the Na^+-K^+-ATPase in isolated bovine articular chondrocytes; molecular evidence for multiple alpha and beta isoforms. *Cell Biol Int* 21: 201–212, 1997.
31. **Mozhayeva MG and Mozhayeva GN.** Evidence for the existence of inositol (1,4,5)-trisphosphate- and ryanodine-sensitive pools in bovine endothelial cells. Ca^{2+} releases in cells with different basal level of intracellular Ca^{2+}. *Pflügers Arch* 432: 614–622, 1996.
32. **Nilius B and Droogmans G.** Ion channels and their functional role in vascular endothelium. *Physiol Rev* 81: 1415–1459, 2001.
33. **O'Brien WJ, Lingrel JB, and Wallick ET.** Ouabain binding kinetics of the rat alpha two and alpha three isoforms of the sodium-potassium adenosine triphosphate. *Arch Biochem Biophys* 310: 32–39, 1994.
34. **Otun H, Aidulis DM, Yang JM, and Gillespie JI.** Interactions between inositol trisphosphate and Ca^{2+} dependent Ca^{2+} release mechanisms on the endoplasmic reticulum of permeabilised bovine aortic endothelial cells. *Cell Calcium* 19: 315–325, 1996.
35. **Owsianik G, Talavera K, Voets T, and Nilius B.** Permeation and selectivity of trp channels. *Annu Rev Physiol* 68: 685–717, 2006.
36. **Pallone TL and Silldorff EP.** Pericyte regulation of renal medullary blood flow. *Exp Nephrol* 9: 165–170, 2001.
37. **Pallone TL, Silldorff EP, and Cheung JY.** Response of isolated rat descending vasa recta to bradykinin. *Am J Physiol Heart Circ Physiol* 274: H752–H759, 1998.
38. **Pallone TL, Zhang Z, and Rhinehart K.** Physiology of the renal medullary microcirculation. *Am J Physiol Renal Physiol* 284: F253–F266, 2003.
39. **Pedersen SF, Owsianik G, and Nilius B.** TRP channels: an overview. *Cell Calcium* 38: 233–252, 2005.
40. **Reuter H, Henderson SA, Han T, Matsuda T, Baba A, Ross RS, Goldhaber JI, and Philipson KD.** Knockout mice for pharmacological screening: testing the specificity of Na^+-Ca^{2+} exchange inhibitors. *Circ Res* 91: 90–92, 2002.
41. **Rhinehart K, Handelsman CA, Silldorff EP, and Pallone TL.** ANG II AT2 receptor modulates AT1 receptor-mediated descending vasa recta endothelial Ca^{2+} signaling. *Am J Physiol Heart Circ Physiol* 284: H779–H789, 2003.
42. **Rhinehart K, Zhang Z, and Pallone TL.** Ca^{2+} signaling and membrane potential in descending vasa recta pericytes and endothelia. *Am J Physiol Renal Physiol* 283: F852–F860, 2002.
43. **Rhinehart KL and Pallone TL.** Nitric oxide generation by isolated descending vasa recta. *Am J Physiol Heart Circ Physiol* 281: H316–H324, 2001.
44. **Shimizu H, Borin ML, and Blaustein MP.** Use of La^{3+} to distinguish activity of the plasmalemmal Ca^{2+} pump from Na^+/Ca^{2+} exchange in arterial myocytes. *Cell Calcium* 21: 31–41, 1997.
45. **Shmygol A and Wray S.** Modulation of agonist-induced Ca^{2+} release by SR Ca^{2+} load: direct SR and cytosolic Ca^{2+} measurements in rat uterine myocytes. *Cell Calcium* 37: 215–223, 2005.
46. **Silldorff EP and Pallone TL.** Adenosine signaling in outer medullary descending vasa recta. *Am J Physiol Regul Integr Comp Physiol* 280: R854–R861, 2001.
47. **Tanaka H, Nishimaru K, Aikawa T, Hirayama W, Tanaka Y, and Shigenobu K.** Effect of SEA0400, a novel inhibitor of sodium-calcium exchanger, on myocardial ionic currents. *Br J Pharmacol* 135: 1096–1100, 2002.
48. **Viatchenko-Karpinski S and Gyorke S.** Modulation of the Ca^{2+}-induced Ca^{2+} release cascade by beta-adrenergic stimulation in rat ventricular myocytes. *J Physiol* 533: 837–848, 2001.
49. **Wang X, Reznick S, Li P, Liang W, and van Breemen C.** Ca^{2+} removal mechanisms in freshly isolated rabbit aortic endothelial cells. *Cell Calcium* 31: 265–277, 2002.
50. **Xie Z and Cai T.** Na^+-K^+-ATPase-mediated signal transduction: from protein interaction to cellular function. *Mol Interv* 3: 157–168, 2003.
51. **Yuan Z, Cai T, Tian J, Ivanov AV, Giovannucci DR, and Xie Z.** Na-K-ATPase tethers phospholipase C and IP3 receptor into a calcium-regulatory complex. *Mol Biol Cell* 16: 4034–4045, 2005.
52. **Zhang Z, Cao C, Lee-Kwon W, and Pallone TL.** Descending vasa recta pericytes express voltage operated Na^+ conductance in the rat. *J Physiol* 567: 445–457, 2005.
53. **Zhang Z and Pallone TL.** Response of descending vasa recta to luminal pressure. *Am J Physiol Renal Physiol* 287: F535–F542, 2004.