Membrane potential controls calcium entry into descending vasa recta pericytes

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Calcium influx through voltage-gated channels mediates contraction of many types of smooth muscle (11, 29). In the kidney, it is generally accepted that afferent arteriolar smooth muscle responds to ANG II stimulation by depolarizing and activating L-type calcium channels. Studies in the efferent arteriole have yielded mixed results, most often failing to identify a role for depolarization and activation of voltage-gated calcium entry pathways (1, 2, 4, 7, 10, 14–17). Hansen et al. (9) recently shed new light on this subject by identifying expression of both L- and T-type calcium channels in juxtamedullary but not cortical efferent arterioles. Also, we recently showed that DVR pericytes respond to ANG II by depolarizing (23, 35), an event that is expected to presage voltage-gated calcium entry into the pericyte cytoplasm. In this study we tested whether vasoconstriction and vasodilation of DVR is accompanied, in parallel, by depolarization and hyperpolarization of pericyte membrane potential. The results confirm that depolarization by K\(^+\) channel blockers or high KCl induces mild vasoconstriction and that the vasodilators pinacidil and bradykinin repolarize ANG II-treated pericytes. Finally, a role for voltage-gated calcium entry pathways was confirmed because the L-type calcium channel blocker diltiazem relaxes ANG II- or KCl-constricted DVR and prevents ANG II-induced calcium entry into the pericyte cytoplasm.

METHODS

Isolation of DVR. Kidneys were harvested from Sprague-Dawley rats (70–150 g; Harlan) that had been anesthetized by an intraperitoneal injection of thiopental (50 mg/kg body wt). Tissue slices were placed in buffer and maintained at 4°C. Individual DVR were dissected from outer medullary vascular bundles and transferred to the stage of an inverted microscope for fluorescence imaging or microperfusion studies as previously described (22–24, 27). The solution used for dissection, microperfusion, and measurement of vasoreactivity contained (in mM) 140 NaCl, 10 Na acetate, 5 KCl, 1.2 MgSO\(_4\), 1.2 Na\(_2\)HPO\(_4\), 1 CaCl\(_2\), 5 HEPES, 5 l-alanine, 5 d-glucose, and 0.5 g/dl bovine albumin. The pH was adjusted to 7.55 at room temperature using NaOH to yield a pH of ~7.4 at 37°C. When patch clamp studies were performed, tissue was stored in physiological saline solution (PSS).

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also served as the extracellular solution during membrane potential recordings. PSS (in mM) 145 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 10 glucose, pH 7.4 at room temperature.

**Isolation of pericytes from the DVR wall.** Small wedges of renal medulla were separated from kidney slices by dissection and transferred to CaCl₂-free PSS containing collagenase 1A (0.45 mg/ml, Sigma), protease XIV (0.4 mg/ml, Sigma), and albumin (1.0 mg/ml) (23, 35). These were incubated at 37°C for 22 min and then transferred back to CaCl₂ (1 mM) containing PSS and held at 4°C in a petri dish. At intervals, vessels were isolated from the digested renal tissue by microdissection and transferred to a perfusion chamber on an inverted microscope (Nikon Diaphot). In the chamber, DVR were captured and aspirated into a microperfusion-suturing system using 10 HEPES, pH 7.2, and nystatin (100 μg/ml with 0.1% DMSO) in ultrapure water. Nystatin in DMSO was kept frozen at −20°C and renewed weekly. Each day, the nystatin stock was thawed, dispensed into the Kaspartate pipette solution at 37°C by vigorous vortexing for 1 min, and subsequently protected from light. Pipettes were backfilled with nystatin-containing electrode solution via a 0.2-μm filter.

Ψm was measured using a CV201AU headstage and Axopatch 200A amplifier (Axon Instruments, Foster City, CA) in current clamp mode (I = 0) at a sampling rate of 10 Hz. Ψm was recorded with pipettes of 8–15 MΩ resistance. Lower resistance pipettes proved technically difficult to use and led to premature loss of seals. Pipettes with nystatin-containing electrode solution were inserted into the bath under positive pressure, positioned near the cell, and the offset of the amplifier was adjusted to null the junction and electrode potentials. The final approach to the cell was controlled with a piezolectric drive (Burleigh PCS-5000). Gigaseals were established by pressing the pipette tip against the cell and applying light suction. After seal formation, the appearance of the cell capacitance transient and the access resistance were monitored using a Digidata analog-to-digital converter and Clampex 7.0 (Axon Instruments, Union City, CA) with 10-mV pulses at a holding potential of −70 mV. Final access resistance was generally between 15 and 40 MΩ. A 3 M KCl agar bridge was used as the bath electrode. Junction and Donnan potential corrections were applied as previously described (22).

**Reagents.** ANG II, bradykinin (BK), probenecid, pinacidil, diltiazem, ionomycin, bovine serum albumin (A2153, Cohn fraction V), nystatin, collagenase 1A, and protease XIV were from Sigma (St. Louis, MO). ANG II, BK, pinacidil, and diltiazem were stored in water in 200-μl aliquots at −20°C and diluted 1:100 or 1:1,000 on the day of the experiment. The enzyme digestion solution was prepared in 50-ml flasks, frozen in 2-ml aliquots, and thawed daily as needed. Fura-2 (Molecular Probes) was stored at 1 mM in anhydrous DMSO. Reagents were thawed once and the excess was discarded at the end of the day.

**Statistics.** Data in the text and figures are given as means ± SE. The significance of differences between means was calculated using Student’s t-test (paired or unpaired, as appropriate) and analysis of variance. Where sampling rates were high, the majority of error bars were suppressed to clarify display of data.

**RESULTS**

**Vasoconstriction by K⁺ channel blockers.** We verified that the K⁺ channel blockers BaCl₂ and TEACl were effective to depolarize DVR pericytes. During I = 0 current clamp recording of Ψm, BaCl₂ (1 mM) or TEACl (30 mM) was introduced into the bath for 5 min and then washed out. BaCl₂ depolarized pericytes from −56.2 ± 2.8 mV to −36.0 ± 4.5 mV (n = 7, P < 0.05, Fig. 1, A and B). TEACl depolarized pericytes from −62.0 ± 2.4 to −35.8 ± 4.3 mV (Fig. 1, C and D, n = 7, P <
Having established that these agents depolarize the pericyte cell membrane, we tested whether they would also constrict in vitro-perfused DVR. After 2 min of baseline recording, either BaCl₂ (Fig. 2A) or TEACl (Fig. 2B) was added to the bath for 10 min and then washed out. BaCl₂ decreased luminal diameter from 12.9 ± 0.8 μm to a minimum of 11.9 ± 0.6 μm, whereas TEACl constricted from 13.4 ± 0.6 to 11.6 ± 0.6 μm. Despite similar degrees of depolarization (Fig. 1, B and D), TEACl was a more effective constrictor of DVR than BaCl₂ (P < 0.05, 6–14 min, %constriction, ordinate, Fig. 2, A and B). We speculate that TEA was a better constrictor because Ba²⁺ can compete for Ca²⁺ influx pathways and Ca²⁺-dependent intracellular signaling processes. Subsequent to washout of BaCl₂ and TEACl, to verify contractility of the vessels and compare their effectiveness to a biological constrictor, ANG II (10⁻⁸ M) was added to the bath. ANG II reduced luminal diameter to 8.6 ± 0.5 and 8.7 ± 0.5 μm for BaCl₂ and TEACl groups, respectively. Thus both BaCl₂ and TEACl constricted DVR but were substantially less effective than ANG II.

KCl-induced depolarization of DVR. To achieve pericyte membrane depolarization exceeding that resulting from BaCl₂- or TEACl-induced K⁺ channel blockade (Fig. 1), we tested the effect of raising extracellular KCl concentration to 100 mM. As expected, this maneuver strongly depolarized the pericytes from -58.2 ± 2.5 to -12.5 ± 0.2 mV (Fig. 3, A and B). We previously showed that ANG II depolarizes DVR pericytes toward the expected equilibrium potential for Cl⁻ ion, about -33 mV (see below) (23). Despite the fact that KCl depolarized pericytes to a greater extent than ANG II, it was less effective in constricting in vitro-perfused DVR.
DVR (Fig. 3C). In separate experiments we tested the hypothesis that depolarization by 100 mM KCl would increase pericyte \( [\text{Ca}^{2+}]_i \). Again, KCl depolarization increased pericyte \( [\text{Ca}^{2+}]_i \), but did so less effectively than ANG II (Fig. 3D). The effect of KCl to depolarize \( \Psi_m \) occurs more rapidly in Fig. 3A than the effect to vasoconstrict or increase calcium (Fig. 3, C and D). This is likely to be related to differences in the rate of bath exchange that, with our apparatus, is faster for patch clamp studies than for videomicroscopy or fluorescent microscopy.

Vasodilation and membrane repolarization by pinacidil and BK. If voltage-gated \( \text{Ca}^{2+} \) entry pathways exist in DVR pericytes, agents that hyperpolarize the cells should be vasodilators. To test this hypothesis, we examined the effects of the \( K_{\text{ATP}} \) channel opener pinacidil. When this agent was applied to the bath of DVR pericytes in log molar increasing concentrations, \( \Psi_m \) progressively declined from resting levels toward the equilibrium potential of \( K^+ \) ion (Fig. 4), a finding that supports the expression of \( K_{\text{ATP}} \) channels in these cells. We tested whether this agent would both repolarize DVR pericytes and vasodilate in vitro-perfused vessels constricted by ANG II. ANG II (10\(^{-8}\) M) depolarized pericytes from a resting level of \(-63.5 \pm 3.5\) mV to \(-32.0 \pm 1.0\) mV \((n = 9, P < 0.01)\). Addition of pinacidil \((10^{-7} \text{ M})\) to the bath induced \( \Psi_m \) oscillations in all but one cell, examples of which are shown in Fig. 5, A and B. On average, the effect of pinacidil was to repolarize the pericytes to \(-53.7 \pm 4.3\) mV \((P < 0.01, \text{Fig. 5C})\), an effect that was reversible after washout. As anticipated, pinacidil effectively vasodilated ANG II \((10^{-8} \text{ M})\)-preconstricted DVR \((n = 8, \text{Fig. 5D})\).

We previously showed that BK \((10^{-7} \text{ M})\) vasodilates ANG II-preconstricted in vitro-perfused DVR (24). We tested the hypothesis that BK would hyperpolarize the
pericyte cell membrane. BK exhibited a complex biphasic effect on $\Psi m$ and repolarized ANG II (10$^{-8}$ M)-treated pericytes (Fig. 6, A and B). On average, ANG II depolarized pericytes from $-52.6 \pm 2.9$ to $-33.4 \pm 1.5$ mV. BK repolarized pericyte $\Psi m$ to $-60.4 \pm 4.4$ mV (Fig. 6C, $n = 7$, $P < 0.01$).

Evidence for voltage-gated Ca$^{2+}$ entry into DVR pericytes. Having established that DVR vasoreactivity parallels the expected changes in $\Psi m$, we next tested whether the L-type channel blocker diltiazem would induce vasodilation and whether the L-type agonist BayK 8644 would induce constriction. Diltiazem re-
versibly vasodilated in vitro-perfused DVR that had been preconstricted with 100 mM KCl (Fig. 7A) or ANG II (Fig. 7B). Also, as expected for functional expression of L-type calcium channels, BayK 8644 constricted DVR, however, compared with ANG II (10⁻⁸ M), the constriction by BayK 8644 (10⁻⁶ M) was mild (Fig. 8).

In a final series of experiments, we tested whether diltiazem could inhibit the pericyte [Ca²⁺]i transients generated by ANG II (10⁻⁸ M). In the protocol illustrated by Fig. 9A, pericytes isolated by stripping from the abluminal surface of isolated DVR exhibited a classical ANG II-induced peak and plateau increase in [Ca²⁺]i. Diltiazem had little effect on the peak but reduced the plateau phase of the ANG II response. For additional confidence, diltiazem was added to the bath of six of the seven pericyte preparations that constitute the control group in Fig. 9A. Those cells had been exposed to ANG II for 10 min before diltiazem treatment and had reached the plateau phase of the Ca²⁺ transient. Diltiazem reversibly reduced the plateau pericyte [Ca²⁺]i (Fig. 9, B and C).

DISCUSSION

It is generally accepted that Vm plays an important role in the excitation-contraction coupling of vascular smooth muscle. Depolarization of the plasma membrane reduces the electrochemical driving force for calcium entry into the cytoplasm but activates voltage-gated calcium entry pathways to enhance transmembrane conductance. A diverse array of voltage-gated calcium channels has been identified; however, in the cardiovascular system, L type and T type predominate (11, 29). In past studies, investigators have examined the effect of L-type voltage-gated channel blockers on regional blood flow in the kidney. Greater enhancement of medullary than cortical blood flow was frequently observed (3, 6, 8, 18, 34). Whether this is due to effects at the juxtamedullary afferent arteriole, efferent arteriole, or DVR is uncertain.

The high degree of specificity of L-type calcium channel blockers has been exploited in several studies to establish the role of L channels in the modulation of vasomotor tone of resistance vessels in the kidney. In the isolated perfused kidney, diltiazem and amlodipine inhibited pressure-dependent and ANG II-induced vasoconstriction, respectively (15, 16). In the hydronephrotic kidney preparation, nitrendipine was found to preferentially vasodilate preglomerular vessels (arcuate, interlobular, and afferent arterioles) (7). L-type calcium channel blockers also effectively dilate ANG II-preconstricted afferent arterioles in the in vitro-perfused juxtamedullary nephron preparation (2, 10). The demonstration that ANG II depolarizes afferent arteriolar smooth muscle implies that this constrictor could activate L channels in the afferent circulation (14).

In addition to voltage-gated calcium entry pathways, vasoconstrictors can increase [Ca²⁺], via other pathways such as store-operated or receptor-operated channels. To examine functional expression of voltage-gated calcium entry in DVR that had been preconstricted with 100 mM KCl (Fig. 7A) or ANG II (Fig. 7B). Also, as expected for functional expression of L-type calcium channels, BayK 8644 constricted DVR, however, compared with ANG II (10⁻⁸ M), the constriction by BayK 8644 (10⁻⁶ M) was mild (Fig. 8).

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Fig. 7. Reversal of DVR vasoconstriction by diltiazem. A: luminal diameters of in vitro-perfused DVR were monitored before and during exchange of 100 mM KCl into the bath. Subsequently, vehicle (n = 6), diltiazem (10⁻⁷ M, n = 5), or diltiazem (10⁻⁵ M, n = 5) was added from 5 to 10 min and then removed (*P < 0.05 vs. vehicle). B: luminal diameters of in vitro-perfused DVR were monitored before and during exchange of ANG II (10⁻⁸ M) into the bath. Subsequently, diltiazem (10⁻⁸ M, n = 13) or vehicle (n = 9) was added from 6 to 11 min (*P < 0.05 vs. control).

Fig. 8. Constriction of DVR by BayK 8644. Luminal diameters were monitored before and during addition of BayK 8644 (10⁻⁶ M, n = 8), ANG II (10⁻⁸ M, n = 9), or vehicle (n = 8) to the bath. BayK 8644 was a mild constrictor of in vitro-perfused DVR (*P < 0.05 vs. control).
gated calcium channels, the effect of KCl-induced depolarization on vasoreactivity and \([\text{Ca}^{2+}]_i\) has been tested. In the hydropnephrotic kidney preparation, 30 mM KCl constricted afferent arterioles to a much greater degree than efferent arterioles, an effect that was blocked by nifedipine (17). Similarly, diltiazem has been found to block KCl-induced vasoconstriction in isolated perfused arterioles (4). Carmines and colleagues (1) showed that KCl depolarization increased \([\text{Ca}^{2+}]_i\) of afferent but not efferent arteriolar smooth muscle. Recently, the pathways through which ANG II stimulates Ca\(^{2+}\) entry into smooth muscle of the afferent arteriole were examined using ratiometric detection of fura-2. ANG II increased \([\text{Ca}^{2+}]_i\) in both arteriolar segments, but nifedipine inhibited only the afferent response, whereas the receptor-operated Ca\(^{2+}\) channel blocker SKF96365 blocked the increase in efferent \([\text{Ca}^{2+}]_i\) (13). These and other studies established that L-type voltage-gated channels provide a functionally important route for calcium entry into smooth muscle of the afferent arteriole.

The recent study of Hansen and colleagues (9) provides an important new perspective on the channel architecture of juxteduodenal renal resistance vessels. Coexpression of the \(\alpha_1\)-subunit for both L- and T-type calcium channels was identified in afferent arterioles and both efferent arterioles and DVR of the juxteduodenal circulation. In contrast, efferent arterioles arising from superficial glomeruli did not express those channel subunits. The authors found that depolarization by KCl could increase \([\text{Ca}^{2+}]_i\) of both juxteduodenal afferent and efferent smooth muscle. The data in Figs. 7–9 provide similar corroborating evidence for the functional presence of such pathways in DVR pericytes. The incomplete inhibition of ANG II-induced vasoconstriction by diltiazem in Fig. 7 raises the possibility that pathways other than L-type channels, such as T-type or receptor-operated channels, might be present in DVR pericytes.

Despite the small size of DVR pericytes, it has been possible to measure \(\Psi_{ms}\) and cellular currents in those cells (23). It is also possible to examine \([\text{Ca}^{2+}]_i\) transients in DVR pericytes after they have been isolated from endothelial cells by stripping. Without prior isolation of the pericytes from the DVR wall, pericyte \([\text{Ca}^{2+}]_i\) cannot be measured because the adjacent endothelia strongly load fura-2 and obscure the small fluorescent emission that originates from the pericytes (32). In this study we exploited those methods to test the hypothesis that depolarization and voltage-gated \([\text{Ca}^{2+}]_i\) entry into DVR pericytes accompany vasoreactivity. The nonspecific K\(^+\) channel blockers BaCl\(_2\) and TEACl depolarize the pericytes and constrict isolated DVR (Figs. 1 and 2). Similarly, elevation of extracellular K\(^+\) markedly depolarizes pericytes (Fig. 3, A and B) and induces DVR vasoconstriction that is reversed by L-type Ca\(^{2+}\) channel blockade (Fig. 7). In this and a prior study, we showed that ANG II depolarizes pericytes from resting levels that lie between −50 and −65 mV toward the equilibrium potential for Cl\(^−\) ion (Figs. 5 and 6; Ref. 23). Despite the ability of K\(^+\) channel blockade and 100 mM extracellular KCl to depolarize...
pericytes to a similar degree, they are less effective than ANG II to stimulate DVR vasoconstriction. This finding contrasts with observations in afferent arteriolar smooth muscle, where KCl is highly effective to induce vasoconstriction (1, 4). On the basis of the comparison among ANG II, Ba2+, TEEA+, and KCl (Figs. 1–3), it seems likely that ANG II activates signaling events that are not mimicked by nonspecific depolarization. Downstream effects of ANG II receptor occupancy, such as tyrosine kinase activation and receptor transactivation, may be needed to fully activate the pericyte contractile response (19, 30).

In addition to verifying that depolarization induces vasoconstriction, we tested whether ANG II-induced vasoconstriction could be reversed by agents that repolarize the pericyte cell membrane. Pinacidil hyperpolarized resting pericytes and repolarized ANG II depolarized pericytes to a remarkable degree (Figs. 4 and 5). In resting cells, pinacidil (≥10−5 M) reduced Vm to values that approached the equilibrium potential of K+ ion (Fig. 4). After ANG II pretreatment, the average effect of this agent was to repolarize the pericyte to a level that lies below the threshold for activation of either T- or L-type calcium channel activation (Fig. 5C). Pinacidil strongly reversed ANG II-induced vasoconstriction, a finding that is consistent with functional importance of depolarization for ANG II to constrict DVR (Fig. 5D). Given the origin of DVR in the relatively hypoxic renal outer medulla, it is not surprising to have identified a robust effect of KATP channel activation in these vessels. Glybenclamide is a blocker of KATP channels that is widely used as a hypoglycemic agent in the treatment of diabetes. Two prior studies demonstrated that glybenclamide reduces blood flow to the renal medulla (28, 33), suggesting that KATP channel activity exerts tonic vasodilatory effects to preserve medullary blood flow. We previously demonstrated that BK relaxes ANG II-constricted DVR, increases NO production, and increases DVR endothelial [Ca2+], (24, 27, 31). In this study, we verified that this is accompanied by pericyte repolarization to a degree that could inhibit voltage-gated calcium entry pathways (Fig. 6).

A number of investigators has examined the ability of L-type calcium channel blockers to affect renal medullary blood flow. Infusion of diltiazem into the renal interstitium resulted in enhancement of papillary blood flow (18). Similarly, intravenous infusion of verapamil selectively enhanced medullary blood flow (8). Using single vessel videomicroscopy, Yagil and colleagues (34) found an increase in vasa recta blood flow with low rates of infusion of the dihydropyridine blocker CS-905. The effects of calcium channel blockade on medullary blood flow have also been examined in pathological models. Papillary plasma flow increased when verapamil was infused into the renal artery of dogs subjected to caval constriction (3). Treatment of the spontaneously hypertensive rat with nisoldipine enhanced medullary blood flow and sodium excretion (6). Taken together, an ability of calcium channel blockade to increase renal medullary blood flow seems well established. The present findings, coupled with the recent work of Hanssen and colleagues (9), imply that DVR are a likely site of action for L-type calcium channel blockers to induce vasodilation.

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