Murine vasa recta pericyte chloride conductance is controlled by calcium, depolarization, and kinase activity

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Lin H, Pallone TL, Cao C. Murine vasa recta pericyte chloride conductance is controlled by calcium, depolarization, and kinase activity. Am J Physiol Regul Integr Comp Physiol 299: R1317–R1325, 2010. First published August 4, 2010; doi:10.1152/ajpregu.00129.2010.—We used the whole cell patch-clamp technique to investigate the regulation of descending vasa recta (DVR) pericyte Ca^{2+}-dependent Cl^{-} currents (CaCC) by cytoplasmic Ca^{2+} concentration ([Ca]_{cyt}), voltage, and kinase activity. Murine CaCC increased with voltage and electrode Ca^{2+} concentration. The current saturated at [Ca]_{cyt} of ~1,000 nM and exhibited an EC_{50} for Ca^{2+} of ~500 nM, independent of depolarization potential. Activation time constants were between 100 and 200 ms, independent of electrode Ca^{2+}. Repolarization-related tail currents elicited by stepping from +100 mV to varying test potentials exhibited deactivation time constants of 50–200 ms that increased with voltage when electrode [Ca]_{cyt} was 1,000 nM. The calmodulin inhibitor N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7, 30 μM) blocked CaCC. The myosin light chain kinase blockers 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7, 1–50 μM) and 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-9, 10 μM) were similarly effective. Resting pericytes were hyperpolarized by ML-7. Pericytes exposed to ANG II (10 nM) depolarized from a baseline of −50 ± 6 to −29 ± 3 mV and were repolarized to −63 ± 7 mV by exposure to 50 μM ML-7. The Ca^{2+}/calmodulin-dependent kinase inhibitor KN-93 reduced pericyte CaCC only when it was present in the electrode and extracellular buffer from the time of membrane break-in. We conclude that murine DVR pericytes are modulated by [Ca]_{cyt}, membrane potential, and phosphorylation events, suggesting that Ca^{2+}-dependent Cl^{-} conductance may be a target for regulation of vasoactivity and medullary blood flow in vivo.

We previously reported that DVR contraction is regulated by voltage-gated Ca^{2+} entry into pericytes. Specifically, we showed that depolarization gates Ca^{2+} entry (55), that vasoconstrictors (ANG II, endothelin-1, and vasopressin) depolarize the cells (4, 34, 35, 54), and that depolarization is mediated by a combination of Cl^{-} channel activation and K^{+} channel inhibition (4, 34). In particular, ANG II activates an outwardly rectifying Ca^{2+}-dependent Cl^{-} conductance that rapidly depolarizes the membrane toward the equilibrium potential of Cl^{-}. Concomitantly, over a longer time period, K^{+} conductances are suppressed (34). The primacy of Cl^{-} in that scheme has been demonstrated; an increase in the equilibrium potential of Cl^{-} by extracellular anion substitution intensifies DVR contraction, and blockade of Ca^{2+}-dependent Cl^{-} current (CaCC) provides vasodilation (54).

The pivotal role of pericyte Cl^{-} conductance in generation of DVR contraction suggests that it could be a prime target for regulation. In this study, we examined the characteristics of murine DVR pericyte Ca^{2+}-dependent Cl^{-} conductance with respect to Ca^{2+} and voltage dependence and tested its sensitivity to blockade of phosphorylation. Murine CaCC exhibits voltage-dependent activation and is Ca^{2+}-dependent, with an EC_{50} of ~500 nM and activation time constant (τ) between 100 and 200 ms. Moreover, blockers of kinase activity inhibit Ca^{2+}-dependent Cl^{-} conductance and reverse pericyte depolarization by ANG II, suggesting plausible regulation of blood flow through signaling cascades that regulate channel or accessory protein phosphorylation.

METHODS

Tissue harvest and microvessel isolation. All investigations involving animal use were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland. Anesthesia was induced by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). The abdomen was opened and the kidneys were excised, leading to euthanasia by exsanguination. The tissue was harvested from C57BL/6 mice (20–30 g; Charles River), sliced, and stored at 4°C in a physiological saline solution [PSS, in mM: 145 NaCl, 5 KCl, 1 MgCl_{2}, 1 CaCl_{2}, 10 HEPES, and 10 glucose (pH 7.4)] at room temperature. Small wedges of renal medulla were separated from kidney slices by dissection and transferred to Blendzyme 1 (0.27 mg/ml) in high-glucose DMEM (Invitrogen), incubated at 37°C for 40 min, transferred to PSS, and stored at 4°C. At intervals, DVR were isolated from the enzymedigested renal tissue by microdissection under a stereomicroscope and transferred to a perfusion chamber on the stage of an inverted microscope. A micropipette was used to capture the vessels and position them near the entrance region of the chamber. They were oriented so that their axis was perpendicular to the approach of the patch-clamp pipettes. Pericytes were identified on the abluminal surface for formation of patch-clamp gigaseals. Photomicrographs of the vessels and description of the patch-clamp procedure are provided elsewhere (35, 49).

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Whole cell patch-clamp recording. Pericyte membrane potential and whole cell currents were monitored by patch clamp of the cells on isolated vessels at room temperature (34, 35). Recordings were made with a CV201AU headstage and Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Membrane potential was sampled at 10 Hz and whole cell currents at 2 kHz. Electrical access for whole cell current recording was accomplished with ruptured patches using 4- to 8-MΩ-resistance pipettes established on abluminal pericytes of explanted vessels (35). Cell-to-cell coupling is sometimes observed when pericytes are accessed for electrical recording, manifest by prolonged capacitance transients. The underlying endothelium is always highly coupled as a syncytium. We previously characterized gap junction coupling and demonstrated expression of several connexin isoforms in the pericytes and endothelium (49). In the present study, when such coupling was observed in the pericyte, study of the cell was abandoned. The mean cell capacitance \( C_m \) of murine pericytes was virtually identical to that previously documented in the rat \( C_m = 12.2 \pm 0.5 \mathrm{pF}, n = 94 \). Similarly,

Fig. 1. Ca\(^{2+}\) and voltage dependence of murine descending vasa recta (DVR) pericyte Ca\(^{2+}\)-dependent Cl\(^{-}\) current (CaCC). A: pulse protocol used to measure CaCC activation (top left). Pericytes were held at \(-80 \mathrm{mV}\) and depolarized to various test potentials for 1,000 ms. Traces show outwardly rectifying CaCC elicited with electrode Ca\(^{2+}\)/EGTA solutions chosen to yield cytosolic Ca\(^{2+}\) concentration ([Ca]\(_{\text{cyt}}\)) of 20, 100, 200, 500, or 1,000 nM. Horizontal arrows indicate zero current level. B: end-pulse membrane current \( I_m \) normalized to cell capacitance \( C_m \) vs. test potential [membrane potential (V_m)] for [Ca]\(_{\text{cyt}}\) between 20 nM and 10 \mu M. C: summary of end-pulse current vs. [Ca]\(_{\text{cyt}}\) illustrates that EC\(_{50}\) was \(-500 \text{nM}\) independent of activating test potential.

Fig. 2. Ca\(^{2+}\) and voltage dependence of murine CaCC activation. A and B: individual records from which the first 400 ms of data were fit to a single exponential to yield activation time constant (\( \tau \)). Horizontal arrow indicates zero current level. C and D: summaries of activation \( \tau \) vs. pulse potential at electrode [Ca]\(_{\text{cyt}}\) of 200, 500, 1,000 and 10,000 nM.
access resistance in murine pericytes was similar to that previously obtained in the rat, typically 7–20 MΩ (53). Cl− currents were isolated by inhibition of K+ conductance with Cs+, glibenclamide, and tetraethylammonium chloride (TEACl). The pipette solution contained (in mM) 115 CsMeSO4, 30 CsCl, 10 TEACl, 10 EGTA, 10 HEPES, and 2 MgATP (pH 7.2). Free Ca2+ concentration in the electrode, for equilibration with the pericyte cytoplasm, was adjusted by addition of CaCl2 to 10 mmol/l EGTA as determined using the Winmax program (http://www.stanford.edu/~capton/), as previously described (36). The results were cross-checked and agreed well with iterative algebraic calculations using a spreadsheet program based on the approach of Tsien and Pozzan (44). During whole cell Cl− current recordings, the extracellular buffer contained 150 mM NaCl, 10 mM TEACl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 10 mM glucose, and 10 μM glibenclamide (pH 7.4).

In the case of pericyte membrane potential measurements, recordings were performed in current-clamp (I = 0) mode, with nystatin used as the pore-forming agent (perforated patches), as previously described (22, 35). The pipette contained 120 mM potassium aspartate, 20 mM KCl, 10 mM NaCl, 10 mM HEPES 10 (pH 7.2), and nystatin (100 μg/ml with 0.1% DMSO) in ultrapure water. The extracellular solution was PSS (see above). Nystatin was dissolved in DMSO, and the excess was discarded daily. Nystatin stock was dispersed into the potassium aspartate pipette solution at 37°C by vigorous vortexing for 1 min; then it was protected from light and backfilled into pipettes via a 0.2-μm filter. Junction and Donnan potentials were corrected as previously described (30, 35).

**Reagents.** Nystatin, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydrro-1,4-diazepine hydrochloride (ML-7), 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydrro-1,4-diazepine hydrochloride (ML-9), N-[2-[[3-(4-chlorophenyl)-2-propenyl]methylamino)methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide (KN-93), and other chemicals were obtained from Sigma (St. Louis, MO). Liberase Blendzyme 1 was obtained from Roche Applied Science. Blendzyme was stored in 40-μl aliquots of 4.5 mg/ml in water and diluted into high-glucose DMEM on the day of the experiment. Stock reagents were thawed once, and the excess was discarded each day.

**Statistics.** Values are means ± SE. The significance of differences between means was calculated using Student’s t-test (paired or unpaired, as appropriate) and analysis of variance. In some figures, data sampled at 10 Hz were averaged 10 values at a time for display at 1 Hz.

**RESULTS**

**Voltage and Ca2+ dependence.** We quantified the voltage and Ca2+ dependence of the murine DVR pericyte whole cell CaCC. Intracellular cytoplasmic Ca2+ concentration ([Ca2+]cyt) was controlled by dialysis of Ca2+/EGTA-buffered pipette solutions through ruptured patches to achieve nominal values of 20, 100, 200, 500, 1,000, and 10,000 nM. Cells were held at −80 mV and stepped between −90 and +80 mV in 10-mV increments for 1 s at each pulse level. The interpulse interval was 10 s. Cell curvatures elicted at various [Ca2+]cyt (n = 6–9 cells in each group) are shown in Fig. 1A. The trace at [Ca2+]cyt of 10 μM has been omitted for clarity, because it nearly overlaps the data obtained at 1,000 nM. A summary of end-pulse current normalized to cell capacitance as a function of pulse potential is shown in Fig. 1B. Currents were strongly voltage-dependent, increasing with [Ca2+]cyt of 200–10,000 nM. As shown in Fig. 1C, half-maximal activation, estimated by Levenburg-Marquardt fit to the four-parameter Hill equation, Im = yo + a([Ca2+]cyt)^b/([Ca2+]cyt)^b + ([Ca2+]cyt)^b), where yo is the current at [Ca2+]cyt = 0, a and b are constants, and C is the EC50 value of [Ca2+]cyt that yields half-maximal current. The EC50 was ~500 nM at all activating potentials between 0 and +80 mV (485, 558, 579, 519, and 529 nM at 0, 20, 40, 60, and 80 mV, respectively).

**Activation τ.** The rate of CaCC activation after membrane depolarization was quantified by fitting 400 ms of whole cell current following the end of the capacitance transient (Fig. 2A). The data fit well to a single exponential (Fig. 2B). The mean activation τ values so obtained are shown in Fig. 2C ([Ca2+]cyt of 200 nM) and Fig. 2D ([Ca2+]cyt of 500, 1,000, and 1,000 nM). At [Ca2+]cyt of 200 nM, currents were small, and fits to the data were variable, yielding τ between 100 and 200 ms. At higher electrode [Ca2+]cyt of 1,000 nM, exponential fits were more robust and yielded τ in the same range. No relationship of τ to electrode [Ca2+]cyt was observed. Above a pulse potential of 20 mV, there was also no relationship between τ and voltage. In contrast, with electrode [Ca2+]cyt of 1,000 nM, there was some tendency for τ to be lower (faster) when potentials
were between $-10$ and $+20$ mV. Differences between $\tau$ at various potentials failed to achieve significance, except for two pairs: $P < 0.05$ for $-20$ vs. $+80$ mV at $[\text{Ca}]_{\text{CYT}}$ of 500 nM and for $-20$ vs. $+50$ mV at $[\text{Ca}]_{\text{CYT}}$ of 10,000 nM.

Conductance and deactivation $\tau$. To study $\text{Ca}^{2+}$-dependent Cl$^-$ conductance and deactivation following repolarization, “tail current” protocols were executed. In a first series of experiments, CaCC were activated by a uniform step depolarization from the holding potential ($-80$ mV) to $+50$ mV for 800 ms (Fig. 3A). Subsequently, the cells were repolarized to $-70$ to $+10$ mV. The CaCC after the repolarization capacitance transient (arrows in Fig. 3, B and C) was quantified to measure conductance and reversal potential. Whole cell currents were low when electrode buffer contained $[\text{Ca}]_{\text{CYT}}$ of 20 nM but robust when $[\text{Ca}]_{\text{CYT}}$ was increased to 1,000 nM. At high $[\text{Ca}]_{\text{CYT}}$, the expected shift of the reversal potential toward the equilibrium potential of Cl$^-$ occurred, and conductance rose from $1.7 \pm 0.4$ nS at $[\text{Ca}]_{\text{CYT}}$ of 20 nM to $5.5 \pm 1.7$ nS at $[\text{Ca}]_{\text{CYT}}$ of 1,000 nM. In a separate series of experiments, the deactivation $\tau$ was quantified. A tail current protocol (Fig. 4A) was executed with electrode $[\text{Ca}]_{\text{CYT}}$ of 200 nM to obtain threshold activation of the CaCC or with electrode $[\text{Ca}]_{\text{CYT}}$ of 1,000 nM to obtain full activation. Pericytes were depolarized to a conditioning potential (+100 mV, 800 ms) and then repolarized to $+80$ to $-140$ mV in 20-mV increments. The deactivation $\tau$ was calculated by fitting the repolarization currents (Fig. 4B) to a single exponential. As summarized in Fig. 4C, when $[\text{Ca}]_{\text{CYT}}$ was 200 nM to achieve threshold activation, the exponential fits to calculate $\tau$ were somewhat erratic, and a correlation with voltage or $[\text{Ca}]_{\text{CYT}}$ was not apparent. In contrast, when $[\text{Ca}]_{\text{CYT}}$ was 1,000 nM and currents were large, an inverse correlation between voltage and the deactivation $\tau$ was demonstrated (Fig. 4D; $P < 0.05$ vs. $-140$ mV).

Kinase-dependent regulation of CaCC. Regulation of CaCC by phosphorylation has been described previously (18, 42, 48, 51, 52). To test whether the murine DVR pericyte currents are similarly modulated, we examined the ability of kinase inhibitors to alter whole cell currents during repetitive pulse depolarizations. With use of an electrode solution buffered to yield $[\text{Ca}]_{\text{CYT}}$ of 500 nM, whole cell currents were repeatedly elicited at 10-s intervals by depolarization of the cells from the holding potential ($-80$ mV) to $+70$ mV for 1 s. We first tested the effects of the calmodulin (CaM) blocker W-7 (30 $\mu$M). After the baseline was recorded (Fig. 5A), W-7 was introduced into the extracellular buffer and then removed. W-7 and the CaCC blocker niflumic acid (100 $\mu$M) reversibly inhibited CaCC (Fig. 5B). The data obtained from seven cells are summarized in Fig. 5C.

The ability of the putative myosin light chain kinase (MLCK) blocker ML-7 to affect CaCC was also studied with sequential depolarizations (Fig. 6). As shown in Fig. 6, 1–50 $\mu$M ML-7 was highly effective and reversible. Similar results were obtained with ML-9 (10 $\mu$mol/l), which reduced the depolarization-induced CaCC from 814 $\pm$ 99 to 562 $\pm$ 80 pA ($n = 15$ cells, $P < 0.05$; data not shown).

Reversal of ANG II-induced pericyte depolarization by ML-7. We tested whether ANG II (10 nM) depolarizes murine pericytes and whether CaCC inhibition with ML-7 repolarizes the membrane. Membrane potential was continuously recorded using nystatin perforated patches as ML-7 was added to the bath in increasing concentrations. Figure 7A shows a recording...
in which the effect of ML-7 on resting potential was investigated, and Fig. 7B summarizes results for five cells. ML-7, at 1, 10, and 50 μM, reversibly hyperpolarized resting cells from −48 ± 3 mV to −50 ± 4 [P = not significant (NS)], −54 ± 6 (P = NS), and −60 ± 7 mV (P < 0.05 vs. baseline), respectively. As in the rat, murine pericytes always depolarized when exposed to ANG II (35), from a baseline of −50 ± 6 to −29 ± 3 mV (P < 0.01, n = 6). Subsequent introduction of increasing concentrations of ML-7 also repolarized ANG II-stimulated cells (Fig. 7, C and D) to −31 ± 2 (1 μM, P = NS), −39 ± 6 (10 μM, P < 0.05 vs. ANG II alone), and −63 ± 7 mV (50 μM, P < 0.01), values that were often below their original resting potential. The effect of ML-7 on membrane potential reversed after washout (to −40 ± 7 mV, P < 0.01 for washout vs. 50 μM ML-7).

**Blockade of CaCC by KN-93.** CaCC activity in some cells has been found to be affected by CaM-dependent protein kinase II (CaMKII) (2, 18, 29, 46). We tested whether the putative CaMKII inhibitor KN-93 (10 μM) can inhibit DVR pericyte CaCC. In a first series of experiments, using electrode [Ca]_{CYT} of 500 nM and a sequential pulse protocol identical to that illustrated in Fig. 5A, KN-93 was introduced into and then removed from the extracellular buffer. As shown in Fig. 8A, some cells appeared to show reversible inhibition, but the results were inconsistent (P = 0.18 for KN-93 vs. control). In a second series of experiments, we included KN-93 or vehicle in the electrode and extracellular buffer from the time of ruptured patch “break-in.” As illustrated in Fig. 8B, the end-pulse whole cell current was significantly lower in KN-93. Moreover, the tendency for currents to increase for several minutes after break-in was absent in KN-93-treated cells.

**DISCUSSION**

Voltage-gated Ca^{2+} channels (CaV) are functionally present in DVR pericytes (53, 55), and all vasoconstrictors thus far tested depolarize those cells (4, 35, 40). Depolarization, in particular, implicates CaV as an entry route, because, without a voltage-gated increase in Ca^{2+} conductance, a rise in membrane potential diminishes electrochemical driving force for Ca^{2+} entry. Hence, with acceptance of an important role for CaV, delineation of the channel architecture that regulates membrane potential becomes an issue of parallel importance. Pericytes are held at low resting potentials by K conductance and basal, ouabain-sensitive, electrogenic Na/K exchange (3, 34) and depolarized by CaCC activation (35). The functional importance of that depolarization has been verified: raising the Nerst potential of Cl by ion substitution enhances DVR contraction, and, conversely, inhibition of Cl conductance induces vasodilatation (54). Hence, studies to date point...
strongly toward a pivotal role for modulation of Ca\(^{2+}\)-dependent Cl\(^-\) conductance in DVR contraction, so that the regulation of CaCC activity is of fundamental interest. A probable sequence of events following contractile agonist stimulation involves store release of Ca\(^{2+}\) by inositol trisphosphate, leading to a rapid increase of CaCC activity that depolarizes the membrane to gate CaV-mediated Ca\(^{2+}\) entry. Such depolarization may be oscillatory, as CaCC tracks \([\text{Ca}]_{\text{CYT}}\) changes that follow movement of Ca\(^{2+}\) into and out of stores via Ca\(^{2+}\)-ATPase, ryanodine, and inositol trisphosphate receptors, respectively. A detailed mathematical simulation of membrane potential changes and intracellular Ca\(^{2+}\) trafficking based on DVR pericyte properties has been recently provided (12).

To examine the characteristics of CaCC, we studied their behavior when exposed to physiological, as well as supraphysiological saturating, \([\text{Ca}]_{\text{CYT}}\) (1 and 10 \(\mu\)mol/l). We also used voltage clamp to raise membrane potential to high values to activate CaCC and examine rectification. Those efforts revealed strong outward rectification independent of \([\text{Ca}]_{\text{CYT}}\) and \(K_d\) for Ca\(^{2+}\) of \(\sim 500\) nM. The latter is consistent with episodic stimulation via subplasmalemmal Ca\(^{2+}\) release (12, 50).

CaCC, studied in different cell types, show variation of properties. In our hands, conductance of CaCC activated at \([\text{Ca}]_{\text{CYT}}\) thresholds of \(200\) nM and saturated at \(1,000\) nM. The independence of the \(EC_{50}\) for Ca\(^{2+}\) and membrane potential contrasts with observations in other cells where the dependence of activation on Ca\(^{2+}\) declines at high pulse potentials (1, 27, 32). Also, in some preparations, rectification declines at high \([\text{Ca}]_{\text{CYT}}\) (13, 19, 27). In DVR pericytes, outward rectification is strong at all \([\text{Ca}]_{\text{CYT}}\). Some investigators observed a dependence of activation kinetics on voltage and Ca\(^{2+}\) (31, 32), whereas others cite voltage independence at \(<1\) \(\mu\)M Ca\(^{2+}\) (19). In contrast, we observe a lack of dependence on electrode.

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Fig. 6. Blockade of murine CaCC by the myosin light chain kinase inhibitor ML-7. A: pericytes were held at \(-80\) mV and sequentially depolarized to \(+70\) mV for 1,000 ms at 10-s intervals (inset). Concatenated examples ( interpulse intervals omitted) of currents elicited during depolarizations at baseline and during exposure to ML-7 (1, 10, and 50 \(\mu\)M) are shown. B: end-pulse current as a function of time during exposure to ML-7. Inhibition was concentration-dependent and reversible. C: summary of end-pulse current before, during, and after exposure to increasing concentrations of ML-7 (\(n = 6\)). *\(P < 0.05\) vs. baseline (control).

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Ca\textsuperscript{2+} and some tendency for activation to be faster at low pulse potentials. With regard to the latter, at such low potentials (or low electrode Ca\textsuperscript{2+}), CaCC achieve only threshold activation, so that the effects of residual contaminating currents that escape Cs\textsuperscript{+}, TEACl, and glibenclamide inhibition on \(\tau\) calculations might be significant. In contrast, when test potentials exceed 20 mV and currents are large, voltage dependence is absent. A thorough study of this issue would require molecular identification of the channel and accessory proteins, heterologous expression to a high level, and reexamination of voltage and Ca\textsuperscript{2+} dependence when CaCC dominates over a large range of voltage. In contrast to findings related to activation kinetics, deactivation of CaCC in DVR pericytes parallels that of many other cells (13, 31, 32). It follows an exponential decay, the rate of which is dependent on voltage when [Ca\textsubscript{cyt}] is high.

Key information concerning plausible structure and regulation of CaCC has recently emerged. The elusive pursuit of their molecular identity yielded to a triad of reports in which CaCC characteristics were reproduced by expression of TMEM16A, a 1,008-amino acid protein, in heterologous systems (5, 41, 48). TMEM16A has been renamed anoctamin 1, and prior candidates for CaCC identity have fallen into disfavor. These include the classical CIC\textsubscript{a}, which may be an adhesion molecule, and the bestrophins, which may serve to modulate Ca\textsuperscript{2+} sensing within the endoplasmic reticulum (16, 17, 20, 26). Anoctamins do not share homology with other ion channel classes. Of 10 isoforms, Ano1 and Ano2 appear to predominate as cell surface proteins. Their predicted structure includes eight membrane-spanning domains with a putative pore between transmembrane regions 5 and 6. Interestingly, the structure lacks an obvious Ca\textsuperscript{2+}-binding motif. Nonetheless, excision of patches into high-Ca\textsuperscript{2+} extracellular buffer can increase Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} conductance in several preparations (24, 27), including our own (54). Thus it seems likely that Ca\textsuperscript{2+} can directly activate some CaCC via binding to the channel or accessory, membrane-bound protein. The identity of CaCC in the DVR pericyte has yet to be fully established. Phosphorylation-dependent regulation of Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} conductance has been observed by other investigators. A probable role for a CaM-binding (e.g., anoctamin lack an IQ-binding motif) or a CaM-regulated accessory protein is supported by the observation that W-7 was both highly and reversibly effective; it inhibited DVR Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} channel whole cell currents during repetitive depolarizations. Roles for CaMKII regulation of Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} conduc-

![Fig. 7. Repolarization of ANG II-stimulated murine pericytes by ML-7. A and B: example and summary (n = 5) of nystatin perforated-patch recording of pericyte membrane potential at baseline followed by sequential addition of ML-7 to the extracellular buffer at 1, 10, and 50 \mu M. C and D: example and summary (n = 6) of membrane potential recording during exposure to ANG II (10 nM) followed by sequential, concomitant addition of ML-7 at 1, 10, and 50 \mu M. Values are means ± SE; most error bars are suppressed for clarity. ML-7 hyperpolarized resting pericytes and reversibly repolarized ANG II-stimulated cells.](http://ajpregu.physiology.org/)

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channel activity by MLCK, including CaCC activity, has been described (42, 43, 51, 52). Hence, we tested for analogous DVR pericyte CaCC regulation. The results showed that putative MLCK inhibitors potently reduce DVR pericyte CaCC activity; ML-7 and ML-9 were highly effective. Given that MLCK is known to be very selective for phosphorylation of myosin light chain (23), that result must be interpreted with caution. Direct phosphorylation of the Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} channel protein by MLCK may not be the explanation. ML-7 and ML-9 lack specificity (8, 11), and actions involving non-kinase-dependent regulation of the cytoskeleton have been described (6). ANG II-induced depolarization was promptly reversed by ML-7. This is consistent with inhibition of Ca\textsuperscript{2+}-dependent Cl\textsuperscript{-} conductance by ML-7; however, we also recognize that activation of one or more classes of K\textsuperscript{+} channels could explain the results.

**Perspectives and Significance**

In summary, we examined the Ca\textsuperscript{2+} and voltage-dependent regulation of murine DVR pericyte CaCC. Its activity is highly sensitive to membrane potential, [Ca\textsubscript{cyt}]\textsuperscript{2+}, and kinase inhibition by W-7, ML-7, ML-9, and KN-93. The specificity of kinase blockers can be brought into question, and these experiments were performed in vitro by patch-clamp methods that dialyze the cytoplasm and expose the cells to high oxygen tensions and low temperatures. Nonetheless, support for plausible combinations of kinase-, voltage-, and Ca\textsuperscript{2+}-dependent regulation of DVR pericyte CaCC, membrane potential, and contraction seems strong. We conclude that pharmacological manipulation of phosphorylation state might provide a target for preservation of medullary blood flow to protect against ischemic insults.

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**DISCLOSURES**

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

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**Fig. 8. Blockade of murine CaCC by the Ca\textsuperscript{2+}/calmodulin kinase II inhibitor KN-93.** A: end-pulse currents wherein pericytes were held at −80 mV and sequentially depolarized to +70 mV for 1,000 ms at 10-s intervals at baseline and as KN-93 (10 μM) was added to and removed from extracellular buffer. Significant suppression of CaCC was not observed. B: KN-93 (10 μM, n = 8) or vehicle (n = 13) was included in the electrode and extracellular buffer from the time of patch formation and break-in. Currents were recorded with the same pulse protocol and subjected to group comparison. Controls illustrate early activation of CaCC immediately after break-in. KN-93 reduced whole cell current (P < 0.05 vs. control for all except the first and last five pulses) and tended to eliminate the early increase of current following break-in.