Kinetic studies of K-Cl cotransport in cultured rat vascular smooth muscle cells

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INTRODUCTION

Cardiovascular disease (CVD), including atherosclerosis, results in significant morbidity and mortality worldwide (55), and in the United States atherosclerosis accounts for more than 400,000 deaths annually (60, 68). CVD involves buildup of plaques in blood vessel walls restricting blood flow. Normally, blood vessel walls display ordered concentric layers of endothelial cells (intima), smooth muscle cells (media), and fibroblasts (adventitia) (46, 66). Proliferation of vascular smooth muscle cells (VSMCs) and migration from the media to the intima augment enlargement and restructuring of the atherosclerotic lesion in the blood vessel wall (69, 85). Narrowing of blood vessels caused by atherosclerosis is a chronic inflammatory response with recruitment of macrophages, lymphocytes, and platelets from circulating blood (67). All these factors restrict blood flow and significantly increase the likelihood of a heart attack or stroke (57, 68).

Phenotypically, VSMCs are heterogeneous as characterized by their shape, size, motility, and proliferative capacity (64). During growth of the atherosclerotic lesion, VSMCs transition from a contractile or differentiated physiological to a synthetic or dedifferentiated pathological phenotype (15, 62, 71, 79). The term “dedifferentiation” in VSMCs does not imply reversal to an early multipotent stage, but rather a transition from contractile to the highly mitotic synthetic phenotype (15, 34).

Several lines of evidence indicate that electroneutral K-Cl cotransport (KCC) is important for cell proliferation, migration, and vasodilation, suggesting its potential roles in CVD (1, 3, 4, 6, 22–25). KCC mediates the coupled movement of K+ and Cl− ions across the plasma membrane (20, 21, 49) and is encoded by four separate genes of the solute cotransporter family, SLC12A4-7, expressing four protein isoforms, KCC1-4 and their spliced variants that display tissue-specific expression important for cellular ion and volume homeostasis. The expression of KCC was initially documented in erythrocytes (49, 52, 59) and thereafter in various other cell types (3, 4, 32, 37, 50, 63, 87) and that of KCC mRNA and protein in rat VSMCs (22–25, 89, 90).

Therefore, in the present longitudinal study, the hypothesis was explored whether there exists a correlation between cell passage (P) number and different functional parameters of K+ dependent KCC transport or KCC, such as maximum velocity, Vm, relative affinity, Km, for external K+ concentration ([K+]o; using Rb+ as K+ congener) and external Cl− ion concentration ([Cl−]o), and ligand transport cooperativity in different synthetic VSMC phenotypes. Results revealed similar Vm values for the two ions, i.e., stoichiometry of unity, as well as Hill cooperativity coefficients of unity, both increasing with cell passage, and a striking longitudinal difference between the apparent external affinities for the transported ions. Putative implications for structure/function changes are offered for the KCC signalosome, a term used here for the first time for a purported KCC multifunctional protein complex consisting of...
of any combination of its 1, 3, and 4 isoforms present in VSMCs and their docking regulatory protein phosphokinases and phosphatases and other cofactors.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** Rubidium chloride (RbCl) and amid sulfonic acid (H2NSO3) were purchased from Alfa Aesar (Ward Hill, MA). Potassium chloride (KCl), sodium hydroxide (NaOH), magnesium chloride (MgCl2), HEPES, Tris, o-glucose, sucrose, calcium chloride (CaCl2), calcium gluconate, DMEM (low glucose), FBS, 0.25% trypsin, and 70% perchloric acid (PCA) were from Thermo Fisher Scientific (Walham, MA). Penicillin (100 U/ml), streptomycin (100 μg/ml), and cesium chloride (CsCl) were from Life Technologies (Carlsbad, CA). Magnesium gluconate, MOPS, and BSA were from Sigma-Aldrich (St. Louis, MO). A bicinchoninic acid (BCA) protein kit, M-Per protein extraction kit, Halt protease inhibitor, and bumentadine from MP Biomedicals (Solon, OH).

**Animals and preparation of primary rat aortic VSMC cultures.** Smooth muscle cells were extracted from thoracic aortas of white Sprague-Dawley rats weighing ~150–200 g supplied by Wright State University’s Animal Resource Facilities. At least triplicate samples were assayed for culture experiments.

Enzymatic dispersion of rat aortic VSMCs was performed as previously described (6, 90). Aortas were washed three times in MEM, mechanically stripped of fat and connective tissue, and treated with collagenase II for 30 min at 37°C. After mechanical removal of the tunica adventitia, tissue was cut into smaller segments and incubated at 37°C with a trypsin and collagenase II solution to obtain a single-cell suspension, which was centrifuged and resuspended in serum-free MEM, diluted to obtain a final concentration of 4 × 10^6 cells/ml, plated onto 75-cm² tissue culture flasks (T-75), and grown in a fully humidified incubator with 95% O2-5% CO2 at 37°C for 24 h to eliminate fibroblasts. Serum-free MEM was replaced with 10% FBS containing growth medium every 2 days until cells reached 100% confluence. Confluent monolayers were treated with 0.25% trypsin and then split at a 1:4 ratio into 1 new T-75 tissue culture flask, 2 subcultured onto 12-well plates for ion fluxes, or 3 100-cm² petri dishes for protein extraction and Western blotting. Confluent VSMCs at P1–4 were defined as contractile VSMCs, whereas P5–90 as synthetic VSMCs, based on previous phenotype characterization reports (6, 12, 15, 33, 35, 43, 45, 54, 56, 62, 65, 71, 79, 82–84, 88, 90). Even though cells adhered more firmly, the use of poly-d-lysine was avoided because ion transport properties are altered in VSMCs (N. C. Adragna, unpublished data).

**Balanced salt solutions for Rb⁺ transport studies.** All stock solutions were prepared in deionized water. Balanced salt solution (BSS-NaCl) contained 20 mM HEPES-Tris buffer (pH 7.4 at 37°C) consisting of (in mM) 130 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 glucose. The preincubation solution contained 0.1% BSA in BSS, and the flux solution replaced 5 mM K⁺ glucose. The preincubation solution contained 0.1% BSA in BSS, and required replacing preincubation solution with flux solution for a specific time at 37°C. Unless otherwise indicated, ouabain and bumetanide were added to both the preincubation and flux solutions to block the Na⁺/K⁺ pump (NKP) and Na⁺/K⁺/2Cl⁻ cotransporter (NKCC), respectively. NEM was added to stimulate KCC activity (7, 47, 52). Unidirectional Rb⁺ transport was terminated by washing the cells five times with an ice-cold isotonic MgCl2/MOPS-Tris wash buffer. Intracellular Rb⁺ content was determined by extracting Rb⁺ ions with 5% PCA and 4 mM CsCl at 4°C for 15 min, and then measured by flame emission spectrophotometry in a PerkinElmer 5000 atomic absorption spectrophotometer as described elsewhere (7, 90). Total protein content was determined by solubilizing the residual cellular matter with 1 N NaOH and using the BCA protein assay as previously described (25). Rb⁺ influx is expressed in nanomoles per milligram of total protein per unit time.

**Antibodies.** Mouse (ms) anti-α-actin (catalog no. 48938) and rabbit (rb) monoclonal anti-vimentin (catalog no. 5741) were purchased from Cell Signaling (Danvers, MA). Ms anti-β-actin (catalog no. sc-517582) was from Santa Cruz Biotechnology (Dallas, TX), ms anti-desmin (catalog no. 550626) from BD Pharmingen (San Jose, CA), and rb polyclonal anti-apelin receptor (APJ) from EMD Millipore (catalog no. abD43). Secondary horseradish peroxidase (HRP)-coupled antibodies were goat (gt) anti-rb IgG or gt anti-ms IgG L chain-specific and CY3-labeled donkey anti-mouse IgG obtained from Jackson ImmunoResearch Laboratory (West Grove, PA).

**Western blot analysis.** The protocol for Western blot analysis was adapted from former publications (3, 50, 89). The cytosolic proteins desmin, vimentin, and α-actin were resolved in 8.5% SDS-PAGE before transfer to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 10% nonfat dry milk or 5% BSA in Tris-HCl-buffered saline tween (TBS-T) for 1 h at room temperature (RT) and then exposed overnight at 4°C to the following primary antibodies: ms anti-α-actin (1:1,000), rb anti-vimentin (1:1,000), ms anti-desmin (1:1,000), and ms anti-β-actin (1:1,000) as an internal positive control. The PVDF membranes were then washed extensively in TBS-T and incubated for 2 h with the appropriate HRP-conjugated secondary antibodies against ms or rb IgG. Membranes were washed three times in TBS-T before signal detection with SuperSignal West Pico PLUS Chemiluminescence substrate (Thermo Scientific) in a Fuji LAS300 CCD camera under high-sensitivity/resolution settings. Densitometry of the protein signals was analyzed by computer software attached to a Kodak camera and/or J software.

**Immunofluorescence of cytoskeletal markers.** The immunofluorescence procedure was described elsewhere (3, 50). VSMCs were seeded in eight-well chamber slides (Laboratory-Tech; NUNC) at a density of 8 × 10^4 cells/well and grown until 25% confluence. Cells were washed with ice-cold 1× PBS, fixed with 0.5 mL/well of 4% paraformaldehyde+saponin for 30 min at 4°C, then incubated in 0.5 mL/well of 3% normal goat serum (NGS) for 1 h at 4°C to block nonspecific immune staining. Cells were incubated with ms primary monoclonal anti-α-actin (1:250) IgG antibodies in 3% NGS overnight at 4°C. Cells were then washed twice with ice-cold PBS then incubated with a sheep anti-ms IgG-FITC (1:1,000) in NGS for 2 h RT followed by final washes in PBS and deionized water. After growth chamber removal, a coverslip was mounted on the slide with Vectashield containing the nuclear stain 4',6-diamidino-2-phenylindole (DAPI). Slides were imaged with an inverted Nikon E400 epifluorescence microscope using ×100 (oil) objectives, images were collected with a Cannon Rebel Model T1i camera system, the background was adjusted and then superimposed using GIMP software.

**Statistical analysis.** GraphPad Prism 5 software (La Jolla, CA) was utilized to determine statistical significance for the increase or decrease in protein expression level (expressing means ± SD). For transport assays, bar or line graphs were created using Origin 7.0 (Origin Laboratories, Northampton, MA) and STATISTIX 7 software (Analytical Software, Tallahassee, FL), and data are reported as means ± SD or SE for N = number of independent experiments and n = number of individual determinations. Statistical analysis was
done by one-way ANOVA followed by a post hoc Tukey test and unpaired Student’s t-test, with significance considered at \( P < 0.05 \).

**RESULTS**

**Growth rate of contractile and synthetic VSMCs as a function of passage number.** Cultured VSMCs were chosen as a model system to uncover further characteristics of their phenotypic differences that may be a useful contribution to our understanding of the progression of CVD (70). Several earlier reports correlated VSMCs’ passage number with their respective phenotypes. At P0–4, VSMCs are defined as predominantly contractile, at P5 as intermediate, and at P6 and later as synthetic (12, 43, 54). An important factor in characterizing VSMC properties is the time required for their proliferation in culture. When propagated at a constant seeding density, an inverse correlation between time to reach confluence and VSMC P number was observed. In accordance, Fig. 1 classifies P0–4 VSMCs as contractile (C) when reaching confluence after 10–14 days, P5–19 as early (E) synthetic between 4 and 7 days, P20–69 as medium (M), and P70–90 as late (L) synthetic phenotype cells consistently in 3 and 2 days, respectively. The statistics for the inset in Fig. 1 and accompanying legend define the contractile, early, medium, and late synthetic phenotypes.

**VSMC phenotype determination by selective markers.** VSMCs are known to change their phenotypes both in vivo and in vitro, and their transition from contractile to synthetic phenotypes can be characterized using selective protein markers (e.g., \( \alpha \)-actin, desmin, and vimentin) that are either up- or down-regulated depending upon the cellular differentiation state (12, 15, 29, 33, 35, 43, 54, 62, 65, 71, 79, 82–84, 88). Also, in cultured VSMCs loss of protein kinase G after passage 4 is associated with transition from contractile to synthetic cells (12, 25, 54). Reversal to a contractile phenotype occurs only upon transfection with PKG-\( \alpha \) cDNA or its active catalytic domain (12, 54).

In the late synthetic VSMCs phenotype (≥ passage 70), there was a 60% reduction in \( \alpha \)-actin (Fig. 2A), 25% in vimentin (Fig. 2B), and 90% fall in desmin protein expression (Fig. 2C) in E versus L synthetic, respectively. Commensurate with the decrease in \( \alpha \)-actin seen in Fig. 2A, cytoplasmic immunofluorescence of filamentous \( \alpha \)-actin expression was abundant in early passage VSMCs (Fig. 2D), whereas \( \alpha \)-actin was redistributed or rearranged in a punctate fashion in higher passages (Fig. 2E).

**\( K^+ \) influx pathways in VSMCs.** Rubidium (\( {}^{85}\text{Rb}^+ \)) has been widely used as a potassium (\( K^+ \)) congener in ion flux studies of rat VSMCs (6, 90), rat C6 glioma cells (31), and sheep red blood cells (4, 21, 52). It is well known that VSMC have a sizable “leak flux” through ion channels (18) that, in our past work and in contrast to low ion channel activity in unaltered red blood cells (49), always interfered with facile measurements of \( \text{Rb}^+ \) through KCC, a variable fraction of the leak flux. Furthermore, the relative ouabain-insensitive \( \alpha \)1 Na-K-ATPase isozyme dominates in rat VSMCs (11). Thus, to rule out these issues potentially affecting a more detailed kinetic study of KCC activity in VSMCs, the concentrations of ouabain and bumetanide to block \( \text{Rb}^+ \) influx through NKCC and NKCC, respectively, were assessed. Figure 3A displays that 2 mM ouabain achieved maximal inhibition of the OS-sensitive (OS) NKCC flux, which, at zero ouabain, was ~50% of the entire \( \text{Rb}^+ \) influx, the remaining 40–50% constituting the ouabain-insensitive (OI) \( \text{Rb}^+ \) influx through NKCC, and the leak component containing KCC. An estimate of the IC\textsubscript{50} yielded ~0.5 mM ouabain, suggesting the presence of predominantly \( \alpha \)1 catalytic subunits. Figure 3B shows that, with 2 mM ouabain blocking NKP, 1 \( \mu \)M bumetanide asymptotically abolished 70% of the OI flux remaining (Fig. 3A), consistent with a high affinity (\(~10^{-7} \text{M}\)) of NKCC1 for this drug (40, 41). The remainder of the uninhibited \( \text{Rb}^+ \) influx constituted influx components through both ion channels and KCC. Thus the optimal ouabain and bumetanide concentrations used here were 2 mM and 2 \( \mu \)M, respectively.

A further necessity to determine \( \text{Rb}^+ \) uptake through KCC was to measure the former in \( \text{Cl}^- \) and \( \text{SF}^- \)-based media and calculate the difference (i.e., the \( \text{Cl}^- \)-dependent \( \text{Rb}^+ \) uptake). Figure 4A shows the \( \text{Rb}^+ \) influx, measured at initial velocity over 30 min, in \( \text{Cl}^- \) (black column) and \( \text{SF}^- \) (grey column). The calculated \( \text{Cl}^- \)-dependent, KCC-mediated influx, 1 nmol/mg \( \times \) min (white column), was ~70% of the total \( \text{Rb}^+ \) influx in \( \text{Cl}^- \) in the presence of 2 mM ouabain and 2 \( \mu \)M bumetanide, which agrees with the data in Fig. 3B. A final test, evidencing \( \text{Cl}^- \)-dependent \( \text{Rb}^+ \) influx via KCC, was to determine its volume and NEM activation (4, 6, 27). As shown in Fig. 4B, KCC activity under hypotonic stimulus more than doubled (with respect to isotonic control, \( P < 0.01 \)), and 0.05 mM NEM activated KCC by threefold (\( P < 0.001 \)). The latter mode of activation, initially discovered by us in erythrocytes, has since become a diagnostic hallmark for the presence of KCC activity, ascribed earlier to thiol group modification (47, 52).

**KCC kinetic parameters in synthetic VSMCs.** Previous studies from this laboratory have focused on characterizing the
activity and expression of the different KCC isoforms as well as their regulation in contractile or early synthetic VSMCs (2–4, 6, 7, 22, 25, 89, 90). The focus of this study was to determine the relationship between KCC activity and VSMCs’ phenotypic transition. Due to the rather extensive manipulations mandated by protocol, VSMCs frequently detached from their plates. Poly-d-lysine, commonly employed to improve cell attachment in culture, changes their ion transport properties and hence was not used to improve cell attachment.

Figure 5 compares the Cl\(^{-}\)/H\(^{+}\)-dependent Rb\(^{+}\)/H\(^{+}\) flux in medium synthetic cells as a function of [Rb\(^{+}\)/H\(^{+}\)]\(_{o}\) at a fixed Cl\(^{-}\)/H\(^{+}\) concentration ([Cl\(^{-}\)/H\(^{+}\)]\(_{o}\) ~145 mM) and Fig. 6 as a function of varying [Cl\(^{-}\)]\(_{o}\).

Fig. 2. Selective protein markers of vascular smooth muscle cell (VSMC) phenotypes. Western blots of two protein extractions from early (E, P6), medium (M, P38), and late (L, P74) synthetic VSMCs. Membranes were probed for α-actin, vimentin, and desmin and then reprobed for β-actin as an internal control. A: ~43-kDa α-actin. B: ~57-kDa vimentin. C: ~53-kDa desmin in P6 and P74 synthetic cells, only. Densitometric quantification of protein bands in A–C was normalized to β-actin (~43 kDa, as internal control), and protein bands of M and L phenotypes to basal expression levels of E cells; average densitometric data of two gels ± range. D and E: immunofluorescence of FITC-labeled α-actin and DAPI-labeled nuclei in P6 and P89 synthetic VSMCs. Magnification ×400, oil.

Fig. 3. Ouabain and bumetanide-dependence of Rb\(^{+}\)/H\(^{+}\) influx in rat vascular smooth muscle cells (VSMCs) through the Na\(^{+}\)/K\(^{+}\)/Cl\(^{-}\) cotransporter (NKCC), respectively. A NLS Lorentzian function was adapted to data in both panels. A: Rb\(^{+}\)/H\(^{+}\) influx measured as a function ouabain (0–2 mM) to obtain maximum inhibition of NKP = ouabain-sensitive (OS) flux with an ~IC\(_{50}\) for ouabain of 0.5 mM. Remaining ouabain-insensitive (OI) flux (interrupted line) constitutes NKCC + KCC + leak flux. B: rat VSMCs with NKP fully inhibited by 2 mM ouabain (see A) were exposed to 0–1 μM bumetanide to maximally inhibit NKCC. The approximate IC\(_{50}\) was 10\(^{-7}\) M bumetanide (interrupted marcation lines). The remaining flux constitutes KCC + leak flux via ion channels. Data shown are one representative experiment done in quadruplicate (\(n = 4\)), from a total \(N = 3\) ± SE.
Fig. 4. Bar graph on the presence of baseline and maximally activated K-Cl cotransport (KCC) in early vascular smooth muscle cell (VSMC) passages. A: baseline KCC in isosmotic media in P5 VSMCs. Rb⁺ influx in Cl (black), Sf (gray), and of KCC (white), the mathematical difference between the former two. Data are the means ± SD of a representative experiment done in quadruplicate (n = 4). B: maximal activation of KCC by hypotonicity and N-ethylmaleimide (NEM) in P3 VSMCs, respectively, normalized to baseline. Percent (%) Cl⁻-dependent Rb⁺ influx (KCC) in isotonic (control; 300 mosmol/kgH₂O) and hypotonic (120 mosmol/kgH₂O) media and after exposure to the thiol-modifying agent NEM (isotonic; 300 mosmol/kgH₂O). NaCl was used to vary the osmolality. Ouabain and bumetanide were present in both preincubation and flux solutions to inhibit NKP and NKCC, respectively; see Fig. 3. Data represent average of two independent experiments, each done in triplicate (n = 3), expressed as means ± SE (n = 6 individual determinations). **p < 0.05 and ***p < 0.01 vs. control group.

Figure 5A reveals saturation for Rb⁺ influx as a function of [Rb⁺]₀ in P42 cells in Cl⁻ and Sf⁻ and for the calculated KCC. Data were plotted by three kinetic approximations: Lineweaver-Burk (LB) plot for (1/KCC vs. 1/[Rb⁺]₀) in Fig. 5B; Hanes-Woolf (HW) plot for ([Rb⁺]₀/KCC) vs. [Rb⁺]₀) in Fig. 5C, and a Hill plot [log KCC/(KCCₘ – KCC) vs. Log [Rb⁺]₀] in Fig. 5D. The correlation coefficients, R, for the linear plots were 0.958, 0.811, and 0.935 respectively, and the significance levels, P, were <0.001, <0.01, and <0.005, respectively. The Vₘ values were 1.6 and 1.7 nmol/(mg protein × min) in Fig. 5, B and C, and the Kₘ/Kₐ values were 17.4, 21.2, and 6.9 mM [Rb⁺]₀, in Fig. 5, B–D, respectively. The Hill coefficient “N” was 1.9, i.e., different from unity (Fig. 5D).

Figure 6A shows a Lorentzian adaptation of the saturating Rb⁺ influx through KCC, corrected for ion channel-mediated Rb⁺ influx, as a function of increasing [Cl⁻]₀, in P39 cells (used above in the Rb⁺-kinetic experiment), i.e., medium at a fixed [Rb⁺]₀ (10 mM) in two unequivocal experiments performed on relatively close passage days, P42 and P39, respectively.

Fig. 5. Kinetic characterization of K-Cl cotransport (KCC) Rb⁺ influx at variable [Rb⁺]₀ in synthetic vascular smooth muscle cells (VSMCs) of P42, 1 of 4 experiments (see also summary in Fig. 7C). A: Rb⁺ influx in Cl⁻ (■) and Sf⁻ (○), and KCC (◆) for the calculated difference between the former. Nonlinear sigmoidal Boltzmann functions (see MATERIALS AND METHODS) were applied to the raw data in A presented as means of quadruplicates ± SD, with a fit correlation R² of 0.98 for Rb⁺ influx in Cl⁻ and Sf⁻ and 0.97 for the calculated KCC. B and C: kinetic constants Kₘ and Vₘ were calculated using the Lineweaver-Burke (LB) and Hanes-Woolf (HW) equations, as indicated in the panels. The R values were 0.958 and 0.81, and the P values <0.001 and <0.01, respectively. D: Hill equation log [Rb⁺]₀ – log Kₐ was applied to obtain 1) the Hill “N” = 1.9 (R = 0.935 and P < 0.005) and 2) Kₐ = 6.9 mM, a measure of the relative Rb⁺ (K⁺) ion affinity. Plotted was log [KCC/KCCₘ – KCC] versus log [Rb⁺], where the x-axis intercept is –log Kₐ for RbCl and KCCₘ = Vₘax of KCC. See DISCUSSION for merit of these kinetic approaches.
synthetic cells. The correlation coefficients, \( R \), of the linear transformation by LB plot of \( 1/KCC \) vs. \( 1/[Cl^-]_o \) (Fig. 6B), HW plot of \([Cl^-]_o/KCC \) vs. \([Cl^-]_o \) (Fig. 6C), and Hill plot \[log KCC/(KCCm – KCC) \] vs. \([Cl^-]_o \) (Fig. 6D) were 0.98, 0.99, and 0.96, and the \( P \) values were < 0.0001 in Fig. 6, B and C and < 0.001 in Fig. 6D, respectively. The \( V_m \) values for KCC were 2.0 and 1.8 nmol/(mg protein \( \times \) min) in Fig. 6, B and C and thus on the order of the \( V_m \) values shown above for Rb\(^+\). The apparent binding affinities for Cl\(^-\) were calculated as 39, 29, and 21 mM for Fig. 6, B, C, and D, respectively. The Hill coefficient \( N \) was 1.6 and thus again different from unity, suggesting cooperativity of KCC-mediated transport.

A summary plot of the KCC influx kinetic results obtained as a function of cell passage measured in eight separate experiments is shown in Fig. 7 which displays all \( V_m \) values (A), the Hill \( N \) coefficients (B), the \( K_m \) values for \([Rb^+]_o \) and \([Cl^-]_o \) calculated by Lineweaver-Burk (LB) and Hanes-Woolf (HW), and Hill analyses, respectively (C and D). Evidently, in Fig. 7A, the \( V_m \) values for Cl\(^-\) dependence of Rb\(^+\) influx via KCC increased by 3-fold over an interval of 80 passages as estimated by both LB and HW analyses, whereas the behavior of the \( V_m \) values for Rb\(^+\) dependence of KCC at higher passages is uncertain due to lack of data. Concomitantly, the Hill coefficient \( N \) rose, at least for Cl\(^-\)-dependent Rb\(^+\) influx, by twofold and seemed to increase moderately also for Rb\(^+\) dependence (Fig. 7B). The \( K_m \) values for \([Rb^+]_o \) (Fig. 7C) remained uninterestingly ~17 mM. In sharp contrast, the \( K_m \) values (Fig. 7D) for \([Cl^-]_o \) were much higher in the early passages, then fell to about one-fourth before rising again by threefold at P86 cells. One-way ANOVA followed by a post hoc Tuckey test on the three data points per passage revealed a statistical difference between the \( K_m \) of P6 vs. P16 (**\( P < 0.01 \)), P6 vs. P39 (*\( P < 0.05 \)), and P86 vs. P16 ([*\( P < 0.05 \), at P16 vs. P86 (\( P < 0.05 \))], and no significance for P6 vs. P86. One-way ANOVA showed no significant difference between the four cell passages for \([Rb^+]_o \).
with previous studies showing similar changes in protein expression as VSMCs transition from contractile to synthetic phenotypes (29, 79, 88). Furthermore, immunocytochemistry (Fig. 2, D and E) revealed early synthetic VSMCs with abundant α-actin protein in a well-organized network, whereas late synthetic VSMCs displayed a severely disorganized and punctate expression of α-actin, suggesting problems with vesicular trafficking and an inability to move to their correspondent loci in the network. Another interpretation is that the cells of late passages are more secretory as their vesicles appear as punctuate and disorganized structures. Indeed, further magnification (not shown) supports this argument. Whether they are more secreting, or the punctuate expression signifies severe disorganization, is hard to discern because an image of the “normal” secretory VSMCs, which exist in equilibrium with the contractile ones in a healthy vessel, was not available. In the latter, the interchange depends on whether the vessel must contract/relax or repair or grow more vessels. Taken together, our operational classification of primary cultured VSMCs into early, medium, and late synthetic phenotypes based on cell passage number is therefore quite appropriate and provides a useful framework to assess the role of KCC in VSMC phenotypic transition.

Attempting to kinetically characterize KCC of VSMCs during their phenotypic expression changes as a function of passage, nonlinear regression analyses (such as a Lorentzian function) were used to describe the raw experimental data (Figs. 5A and 6A), and three linear transformations (Figs. 5, B–D and 6, B–D), each with their own limitations, to obtain estimates of the standard parameters such as \( V_m \), \( K_m \), and cooperativity, Hill \( N \). In the LB approach of Figs. 5B and 6B, a plot of \( \frac{1}{V} = \frac{K_m}{V_mS} + \frac{1}{V_m} \) shows typical data clusters at low \( \frac{1}{[Rb]_o} \) or \( \frac{1}{[Cl]_o} \), i.e., at high \([Rb^+]_o\) or \([Cl^-]_o\), and few data at high \( \frac{1}{[Rb]_o} \) or \( \frac{1}{[Cl]_o} \), i.e., at the low \([Rb^+]_o\) or \([Cl^-]_o\) values close to the presumable KCC affinities for these ions. Any error in the latter two therefore significantly affects the slope of the LB function and thus contributes to the uncertainty of the obtained \( K_m \) and \( V_m \) values. In contrast, the Hanes-Woolf plot of \( \frac{S}{V} = \frac{S}{V_m} + \frac{K_m}{V_m} \) yielded a much more even distribution of the data points in the plot of \( \frac{[Rb]_o}{Rb \text{ influx}} \) or \( \frac{[Cl]_o}{Cl \text{ influx}} \) versus \([Rb^+]_o\) or \([Cl^-]_o\), and thus a greater precision of the parameters \( 1/V_m \) and \( -K_m \) for either ion, which indeed is the case seen in Figs. 5C and 6C. A similar “less weighted” spread of data was obtained applying the Hill equation \( log_y = Nlog[S] - Nlog[K_d] \), where \( S = [Rb^+]_o \) or \([Cl^-]_o\), \( v \) = the observed initial KCC velocities, and \( V_m \) the maximal KCC Rb influx velocity, \( N \) = Hill coefficient of cooperativity, and \( K_d \) = apparent dissociation constant of the ionic ligand, as shown in Figs. 5D and 6D. Inspection of the data given in Figs. 5 and 6 reveal that the \( K_m/K_d \) values were more variable than the \( V_m \) data.

Based on the three independent kinetic approaches, it was inviting to perform statistical analyses on these values as shown in the longitudinal summary in Fig. 7. In Fig. 7A, the \( V_m \) values increased, especially doubled in the case of KCC Rb⁺
influx as function of [Cl\(^-\)]\(_o\) over a wide spread of passages (solid lines). The paucity of data points at higher passages did not permit statistical evaluation. Also, the Hill \(N\) increased, particularly in the case of Rb influx versus function of [Cl\(^-\)]\(_o\) between lower and higher cell passages. The differences in the behavior of the \(K_m\) values calculated for [Rb\(^+\)]\(_o\) and [Cl\(^-\)]\(_o\) by the three approaches are stunning: Based on one-way ANOVA analyses followed by a post hoc Tukey test, the \(K_m\) values for [Rb\(^+\)]\(_o\) were unchanged, but significant differences were found for [Cl\(^-\)]\(_o\). Early P6 cells showed the lowest affinity for Cl\(^-\) (highest \(K_m\) values), which fell to almost one quarter at P16 and then gradually rose to P86, where there was no significant difference vis a vis P6. In *Xenopus* oocytes, the K\(^+\) affinity series for exogenously expressed isoforms is KCC1<KCC2 and KCC3 (32); therefore, the closely related \(K_m\) values for [Rb\(^+\)]\(_o\) are surprising. In NKCC, two amino acids are encoded in the second transmembrane domain of NKCC2, residue 220 for Na\(^+\) and 216 for K\(^+\) ions, respectively, their mutual interaction being affected by [Cl\(^-\)]\(_o\), as proposed by Lindinger's group (10, 30). If this model can be extrapolated to the KCCs, the large variation in [Cl\(^-\)]\(_o\) affinity did not correlate with the [K\(^+\)]\(_o\) affinity as the cell passages progressed. Are the strikingly different \(K_m\) values for [Cl\(^-\)]\(_o\), a function of presumable multiple contact points for Cl\(^-\) binding per individual KCC paralog (78) which possess a different Eisenman anion series (58) for KCC1 ([Cl\(^-\)\(\rightarrow\)SCN\(^-\) = Br\(^-\)\(\rightarrow\)PO\(_4\)\(^{3-}\)\(\rightarrow\)I\(^-)\) as opposed to KCC4 ([Cl\(^-\)\(\rightarrow\)Br\(^-\)\(\rightarrow\)PO\(_4\)\(^{3-}\) = I\(^-)\(\rightarrow\)SCN\(^-\)\(\rightarrow\)I\(^-)\) changing with progressing cell passage, or due to different homo/hetero dimerization of the three KCC paralogs? In this context it may be relevant to recall that intracellular Cl\(^-\) levels, [Cl\(^-\)]\(_i\), respond to [K\(^+\)]\(_o\) and thus modulate serine autophosphorylation of WNK (with no lysine) in renal distal collecting tubules (9, 39). Extrapolating from the low affinity for [Cl\(^-\)]\(_o\) to a similar internally kinetic behavior of KCC may point to modulation of phosphorylation through WNK-controlled SPAK at the transporter's N- or C-terminal serine/threonine residues, a hypothesis that can be tested.

That the much harder to measure affinities for Cl\(^-\), but not for Rb\(^+\), so strikingly changed, reminds one of the landmark review on “Chloride in Smooth Muscle” by Chipperfield and Harper; their classic paper pointed to the overriding presence of Cl\(^-\) channels causing difficulties of measuring Cl\(^-\)-dependent cation pathways (e.g., NKCC and KCC) in smooth muscle cells (18). However, as Figs. 4–6 reveal, as well as the statistical behavior of \(K_m\) values for [Cl\(^-\)]\(_o\) in Fig. 7, special precautions were taken to define KCC in VSMCs. 1) Exposure to hyposmotic solution and to NEM caused up to threefold stimulation of KCC, both hallmark criteria for the presence of this transporter (7, 13, 31, 48, 52), causing its upregulation due to dephosphorylation of key threonine and serine residues within its C-terminal domain (5) by PP1/2 phosphatases (28, 42, 80). We have recently shown how the phosphorylation state of KCC3, for example, confers a potent molecular switch of transporter activity, cell volume, and K\(^+\) content (5). Indeed, the KCCs have been proposed by us in VSMCs (3) and found by others in different cell types, and especially in cancer and cell aging (16, 17), playing important roles in cell proliferation, migration and malignancy. 2) Significantly higher ouabain concentrations (2 mM) were necessary to inhibit NKP activity in rat VSMCs compared with previous reports using different human cell types [e.g., human lens epithelial and embryonic kidney (36, 51)]. This means rat VSMCs primarily express the \(\alpha1\) isoform. In human cells, three main \(\alpha\)-subunits (\(\alpha1\geq\alpha3\geq\alpha2\)) of NKP have similar high ouabain affinities (19) as opposed to rodents, where the \(\alpha2\) isoform has a significantly higher ouabain affinity than \(\alpha1\) (44, 61). We further determined that NKCC activity was fully inhibited with 1 \(\mu\)M bumetanide, consistent with its high affinity for this cotransporter. Altogether, the ouabain-sensitive portion of the total Rb\(^+\) influx (NKP) was ~50%, and NKCC constituted ~40% of the remaining ouabain-resistant Rb\(^+\) influx, leaving 10% for the basal leak that includes ion channel and KCC activity.

How finally can one reconcile an apparent concomitant or simultaneous change of in \(V_m\), Hill \(N\), and \(K_m\) parameters for [Cl\(^-\)]\(_o\) without a translational change in KCC1, 3, and 4 protein expression in VSMCs as a function of cell passage (data not shown)? Regardless, whether the KCC proteins were cytosolic or membrane bound, since their actual mass did not change, the obvious answer is that a greater level of KCC dephosphorylation at the now well-established N- and C-terminal serine and threonine moieties, as reflected in kinetic parameter change, commenced as a function of cell passage (5). It is notable that VSMCs lose their PKC (12, 25, 56), an enzyme that belongs in the lipid signaling orbit in the process of changing from contractile to synthetic phenotypes. An alternate hypothesis cannot be excluded. It has been recently shown that C-terminal domains of prokaryotic *Archea* CCC contain \(\alpha/\beta\) folds, also found in stress proteins, which participate in solution dimer formation governing homodimer structure/function relationships of full-length CCC transporters (86). Perhaps these protein equilibria are affected by the different Eisenman anion series coordinates of the three KCC isoforms 1, 3, and 4 that are not only transcribed in rat VSMCs into mRNA (23–25) but are also translated as proteins (data not shown).

The changes in KCC kinetics summarized in Fig. 7 may be indeed related to various additional mechanisms. For instance, changes in KCC isoforms' expression profile composition in the late and medium synthetic phenotypes, or mutually exclusive KCC gene-expression profiles, may occur in VSMCs, as proposed earlier for the chronic effect of PDGF in early synthetic VSMCs (90). This is an important area of research that is out of the scope of the present study and requires further investigation. In addition, changes and distribution of the cytoskeleton could modulate membrane proteins and alter ion transport activity (38). Because cytoskeletal proteins regulate intracellular signaling cascades, their distribution and location may be important for determining the VSMC phenotype. Thus any reorganization of the cytoskeleton or signaling molecules could affect VSMC phenotypic modulation (88). Moreover, several cell culture models suggest that the cytoskeleton machinery is linked to cell migration (14, 38, 81). Further evidence indicates ion channels and transporters play a significant role in cell migration (3, 16, 73, 74). During the cell migratory process, there is polarized distribution of ion channels and transporters due to structural reorganization of the cytoskeleton, as we previously suggested for VSMCs (3). Based on these findings, it is plausible that, as VSMCs undergo phenotypic transformation, an interaction between KCC and the cytoskeleton could enhance membrane expression and increase its transport activity (3). As discussed above and in support of the role KCC might play in VSMC phenotypic transition, recent studies on
the expression of KCC isoforms in embryogenesis and dedifferentiated cancerous cells has linked KCC to tumorigenesis, cell proliferation, and migration (16, 75–77, 87). Similarly, VSMC synthetic phenotypic transition is also associated with cell proliferation and migration (71). Thus differential expression (22–25, 90) and activity of the KCC isoforms in VSMCs could be driving this phenotype transition. Alternatively, it is also possible that the transition to a medium and, even later, synthetic phenotype may affect KCC regulation.

Summary. Concomitant with the process of transition from contractile to synthetic VSMC phenotypes, as defined here based on the passage numbers, significant changes in kinetic parameters such as \( V_m \), \( K_m \), and Hill \( n \) for the cotransported \( \text{Rb}^+ \) and \( \text{Cl}^- \) ions were detected that may contribute to our understanding of the pathological progression during CVD. These findings point and add to the growing information base that KCC and its tissue-specific isoforms may exert their participation through abnormalities or changes in their kinetic parameters in the development of vascular lesions, leading to vascular occlusion causative for heart attacks and strokes.

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