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K-Cl Cotransport: Membrane Immunofluorescence Localization by Confocal Microscopy of the KCC1 Protein, Before and After Truncation of the COOH-Terminal Domain, in Human Embryonic Kidney (Hek293) Cells

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(Shrode et al. 1995. *Am. J. Physiol.* 269:C257). To understand the role of MLC phosphorylation and contraction in volume-sensitive signaling, we examined the regulation of NKCC1 in vascular smooth muscle. Thoracic aorta was removed from male Sprague-Dawley rats and cleaned of adventitia and endothelium. NKCC1 activity was measured as bumetanide-sensitive efflux of ^{86}Rb after loading for 2 h at 37°C. Isometric force generation was measured with a Kistler-Morse force transducer in an organ bath. Phenylephrine (PE, 10 μM) acutely increased NKCC1 activity 51%. This was not due to cell shrinkage, since it was not prevented by blocking K^+ channels with 5 mM Ba^{2+}, and because similar stimulation of NKCC1 was observed with isosmotic 80 mM KCl. Stimulation of NKCC1 by PE was blocked by ML-9 (63% at 75 μM) and wortmannin (100% at 2 μM), inhibitors of MLC kinase. Stimulation of NKCC1 was also blocked by butanedione monoxime (BDM), an inhibitor of myosin ATPase (58% at 2 mM). The inhibition of NKCC1 corresponded roughly to the inhibition by these compounds of PE-induced isometric force generation (51, 83, and 31% for ML-9, wortmannin, and BDM, respectively). To determine the role of smooth muscle shortening, efflux was measured in aortic segments placed snugly over flexible or rigid tubing. There was a 76% reduction in the stimulation of NKCC1 by PE with rigid tubing as compared with flexible tubing. We conclude that direct activation of MLC kinase, independent of cell volume, stimulates NKCC1 in vascular smooth muscle through contraction and muscle shortening and that this is the mechanism for activation of NKCC1 by vasoconstrictors. These results suggest that NKCC1 is controlled by the contractile state of the cell. (Supported by NIH grants HL 47449, DK50268, and HL54829.)

58. Molecular Cloning of Mouse KCC1 cDNA, Cloning and Mapping of its Gene, and Immunological and Functional Characterization of its Encoded Polypeptide B.E. SHMUKLER, W. SU, M.N. CHERNOVA, L. deFRANCESCHI, C. BRUGNARA, and S.L. ALPER, *Molecular Medicine and Renal Units, Beth Israel Deaconess Medical Center; Department of Laboratory Medicine, The Children's Hospital; Departments of Medicine, Cell Biology, and Pathology, Harvard Medical School, Boston, Massachusetts; and Department of Internal Medicine and General Pathology, University of Verona, Verona, Italy*

KCC1 K-C1 cotransporter mRNA is widely expressed, K-C1 cotransport activity has been measured in a more restricted group of tissues. Detection of endogenous KCC1 polypeptide has not yet been reported. We report here: 1, the molecular cloning and sequencing of the mouse KCC1 (mKCC1) cDNA and partial sequencing of the gene, allowing chromosomal mapping by physical linkage to the LCAT gene; 2, characterization of a CA repeat polymorphism exhibiting mouse strain specificity; 3, characterization of three anti-peptide antibodies that recognize recombinant KCC1 polypeptide expressed in 293 cells and *Xenopus* oocytes; 4, tissue distribution of KCC1 mRNA and protein in mouse; 5, detection of immunoreactive putative KCC1 polypeptide in erythrocytes of species in which cotransport activity has been studied; and 6, demonstration of increased putative KCC1 polypeptide abundance proportionate to reticulocyte count in density-fractionated cells, hemorrhagic reticulocytosis, and sickle cell disease and thalassemia. We also show that mKCC1 expressed in *Xenopus* oocytes replicates the regulatory patterns described in erythrocytes. ^{86}Rb uptake is activated by hypotonic swelling, N-ethymaleimide, and staurosporine, requires extracellular $C1^-$, and is blocked by the serine phosphatase inhibitors calyculin and okadaic acid, and by the diuretic DIOA. These findings and reagents will facilitate future experiment on structure-function relationships of KCC1 and of the pathobiology of KCC1-mediated K-C1 cotransport. (Supported by NIH grants.)

59. K-Cl Cotransport: Membrane Immunofluorescence Localization by Confocal Microscopy of the KCC1 Protein, before and after Truncation of the COOH-terminal Domain, in Human Embryonic Kidney (HEK293) Cells PETER K. LAUF,* KENNETH B.E. GAGNON,* JIN ZHANG,* ERIC DELPIRE,‡ ROBERT E.W. FYFFE,* and NORMA C. ADRAGNA,* **Wright State University School of Medicine, Dayton, Ohio; and ‡Vanderbilt University Medical Center, Nashville, Tennessee*

Coupled movement of K and Cl is mediated by at least two isoforms of the K-Cl cotransporter: KCC1 and KCC2 (Gillen et al. 1996. *J. Biol. Chem.* 271:16237–16244; Payne et al. 1996. *J. Biol. Chem.* 271:16245–16252). Based on the hydropathy plot, there are 12 trans-membrane domains with the NH_2- and COOH-terminal domains (NTD and CTD) assigned to the cytoplasm. Here, we demonstrate by immunofluorescence confocal microscopy the membrane localization of the KCC1 protein in HEK293 cells after transient transfection with the rabbit (rb) full-length cmycKCC1 gene (kindly provided by Drs. Gillen and Forbush). To remove the CTD at the codons below HIS686, we truncated the KCC1 gene at nucleotide position 2011 at the 3′ end of the cDNA (Adragna et al. 1998. *J. Gen. Physiol.* 112:25a). HEK293 cells were grown in culture chamber slides for 48 h. Transient transfection with cmycKCC1 ± CTD was performed with lipofectamine plus (Life Technologies). After 2 d, cells were fixed by p-formaldehyde and exposed to two primary antibodies, separately or in combination: monoclonal mouse anti-cmyc recognizing the cmyc epitope at the NTD, and rb anti–rat (rt) CTD against an epitope within a 77-amino acid sequence (949–1026) of the CTD. Secondary fluorescence-conjugated antibodies were goat anti–mouse TRITC (tetramethylrhodamine-5-isothiocyanate)-IgG (red) and goat anti–rabbit FITC (fluorescein-isothiocyanate)-IgG (green). Images were obtained with an Olympus Fluoview confocal laser scanning microscope. The following findings were made: 1, in nontransfected HEK293 cells, rb anti-rtCTD revealed punctuate (hot spot) membrane labeling, presumably of endogenous KCC1. 2, In full-length cmycKCC1-transfected cells, incubation with rb anti-rtCTD produced membrane localized hot spots, as well as a diffuse fluorescence. Dual labeling with anti-cmyc indicated colocalization of labeling with the two antibodies, and that the labeling was primarily membrane-localized. 3, In cells transfected with the truncated cmycKCC1-CTD cDNA, rb anti-rtCTD revealed hot spots (endogenous KCC1?), but failed to show diffuse green fluorescence, whereas anti-cmyc caused weak diffuse red fluorescence of membrane and cytoplasm. 4, Controls with only secondary FITC- and TRITC-conjugated antibodies were negative. We conclude that by binding to the NH_2- and COOH-terminal epitopes, both rb anti-rtCTD and anti-cmyc antibodies are useful tools for membrane localization of KCC1 proteins encoded by either the full-length or truncated KCC1 cDNAs. (Supported by Ohio Research Challenge Grant 662349 to P.K. Lauf and N.C. Adragna.)