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Significance of the KCCl Cytoplasmic COOH-Terminal Fragment for the pH Dependence of K-Cl Cotransport in Transfected Human Embryonic Kidney Cells

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somes significantly increased ^{86}Rb uptake, but had no effect on ^{36}Cl flux. Taken together, these observations indicate that the channel activity we observe arises from pI_{Cln} rather than contaminant proteins. However, the characteristics of the channel are not consistent with the hypothesis that pI_{Cln} is the $I_{Cl,swell}$ channel. (Supported by NIH grants NS30591 and DK51610.)

65. Functional Demonstration of Na-K-2Cl Cotransporter Activity in Isolated Polarized Choroid Plexus Epithelial Cells

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The choroid plexus (CP) secretes cerebral spinal fluid (CSF) and plays a central role in brain ionic homeostasis, metabolic waste removal, and nutrient transport. The cellular mechanisms of CSF secretion are incompletely understood largely because of the inaccessibility of the CP for cellular and molecular studies. Previous investigations have suggested that the Na-K-2Cl cotransporter is localized to the CP basolateral membrane and that it plays a central role in fluid secretion. However, more recently it has been shown by immunofluorescence that the cotransporter is localized predominantly, if not exclusively, to the apical cell membrane (Plotkin et al. 1997. *Am. J. Physiol.* 272:C173). To investigate Na-K-2Cl cotransporter function and regulation, we developed a dissociated CP cell preparation in which single, isolated cells maintain their normal polarized morphology. A distinct apical pole, marked by a prominent brush border, and a basolateral pole are readily visible by differential interference contrast (DIC) microscopy in living and fixed cells. Immunofluorescence demonstrated that the Na/K ATPase and the Na-K-2Cl cotransporter remain localized to the brush border while the Cl/HCO_3 exchanger (AE2) is confined to the basolateral membrane. We used video-enhanced DIC microscopy and cell volume measurement techniques to investigate Na-K-2Cl cotransporter function. Application of 100 μM bumetanide, a selective inhibitor of the cotransporter, caused CP cells to shrink $-11.8 \pm 4\%$ at an initial rate of $-2.5 \pm 0.9\%$/min ($n = 13$), suggesting that the cotransporter is normally active in isolated cells. Elevation of extracellular K^+ from 3 to 25 mM caused the cells to swell $32.9 \pm 12.5\%$ at $11.8 \pm 3.7\%$/min ($n = 15$). High K^+-induced swelling was blocked completely by Na^+ removal or by addition of 100 μM bumetanide. Taken together, these results suggest that the Na-K-2Cl cotransporter is a major pathway for the entry of K^+ and Cl^- into CP cells and that it may play an important role in the reabsorption of these ions from the CSF. (Supported by NIH grant NS30591.)

66. Significance of the KCC1 Cytoplasmic COOH-Terminal Fragment for the pH Dependence of K-Cl Cotransport in Transfected Human Embryonic Kidney Cells

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We have recently demonstrated functional evidence for an internal pH sensor of erythrocyte K-Cl cotransport (COT) through inhibition by internal protons and diethylpyrocarbonate, and proposed that internal histidines (HIS) may be involved (Lauf and Adragna. 1998. *Cell. Physiol. Biochem.* 8:46–60). This hypothesis is consistent with the presence of the majority of HIS within the COOH-terminal cytoplasmic portion of the KCC1 from human, rabbit (rb), and rat (rt) (Gillen et al. 1996. *J. Biol. Chem.* 271:16237–16244). Thus, truncation of the COOH-terminal cytoplasmic tail of the KCC1 above HIS686, conserved throughout the entire cation–anion cotransporter superfamily, should provide, through measurement of Rb-Cl inward fluxes, a first clue about the functional contribution of this fragment to the hypothetical pH sensor of K-Cl COT. We obtained the rbKCC1-pJB20 expression vector through the courtesy of Dr. B. Forbush (Yale University, New Haven, CT) and created a new KCC1-pJB20 expression vector without the nucleotides expressing the major portion of the KCC1 COOH-terminal fragment (KCC1-CTHIS686). The nucleotides of KCC1 cDNA were cut, with BamHI as the restriction enzyme, between the end of TMD12 and before HIS686 between positions 1981 and 2074 to obtain rbKCC1-CTHIS686. The molecular size of the cDNA was verified by agarose gel electrophoresis, and the new plasmid transfected and expressed in human embryonic kidney cells (HEK293). Expression of the full length and the truncated KCC1 was verified in cellular subfractions by Western blots using a commercially available mouse anti–cmyc antibody against an NH_2-terminal cmyc epitope and a rb antibody against a 70 amino acid peptide of the cytoplasmic COOH-terminal tail of rtKCC1 (kindly provided by Dr. E. Delpire, Vanderbilt University, Nashville, TN). Both KCC1 and KCC1-CTHIS686 reacted positively in a Western blot with mouse anti–cmyc; however, only the full length KCC1 did with rb anti–rt COOH terminus. Whereas the full length KCC1 had a molecular weight of ~160 kD as published (Gillen et al. 1996. *J. Biol. Chem.* 271:16237–16244), the molecular weight of rbKCC1-CTHIS686 was ~100 kD and, like the full length KCC1, appeared in the membrane fraction, suggesting normal posttranslational processing.

HEK293 cells (controls, and transfected with either the full length rbKCC1 or rbKCC1-CTHIS686 cDNA) were equilibrated in media of pH 6–9 and Rb influx was measured between 0 and 6 min at 37°C in the presence of various inhibitors of other K-transporting systems in Cl- and sulfamate-containing media. K-Cl COT is defined as the Cl-dependent Rb influx or Rb-Cl influx; i.e., the difference between Rb fluxes in Cl and sulfamate, measured in flux units (FU) of nanomoles Rb per milligram protein per minute. *N*-ethylmaleimide (NEM) was used to specifically stimulate K-Cl COT (Lauf and Theg. 1980. *Biochem. Biophys. Res. Commun.* 70:221–242). The basal Rb-Cl flux of control HEK293 cells was ~1.5 FU at pH 7 and fell to ~1 FU at pH 6 and 8. Exposure to 0.5 mM NEM stimulated Rb-Cl flux by almost threefold to 4 FUs, with a maximum at pH 7.4 and minima at pH 6 and 8, suggesting the presence of pH-dependent and -independent K-Cl COT. The basal Rb-Cl influx of HEK293 cells transfected with KCC1 was about twice that of nontransfected controls, again with a maximum around pH 7, and minima at pH 6.3 and 8.4. When 0.5 mM NEM was present, Rb-Cl influx was about 10-fold higher with a bell-shaped maximum at pH 7 (40 FUs), and flux minima at pH 6.3 (25 FUs) and 8.4 (5 FUs), suggesting the presence of at least two proton-titratable groups on the NEM-activated carrier, as predicted from work with erythrocytes. After transfection with KCC1-CTHIS686, the basal Rb-Cl influx was still elevated as in the case of KCC1-transfected cells, but the flux was entirely pH independent. After stimulation by NEM, HEK293 cells transfected with KCC1-CTHIS686 revealed flux activities of ~20 FUs across the entire pH range, without the sharp pH 7 activation seen in cells transfected with the full-length gene. Hence, the truncated KCC1 protein is capable of pH-independent K-Cl COT, but apparently fails to develop the maximum bell-shaped response at pH 7.

These data suggest, that (*a*) endogenous as well as KCC1-induced K-Cl COT in HEK 293 cells is composed of pH-independent and

-dependent components, that (*b*) NEM markedly (by an order of magnitude) enhances both, and that (*c*) truncation of the COOH-terminal cytoplasmic fragment of KCC1 (KCC1-CTHIS686) abrogates a characteristic bell-shaped pH response around pH 7 elicited by NEM. Our findings are consistent with the hypothesis that the cytoplasmic COOH-terminal fragment of the KCC1 plays a significant role in the response of K-Cl COT to cellular pH. (We thank Dr. L. Lu, Wright State University, for helping with molecular biology techniques. Supported by NIH DK 37,160, AHA MV-95-01-S and an OHIO Research Challenge Grant.)

67. Contribution of ClC-2 to Intestinal Cl^- Secretion in the Gastrointestinal Tract of Cystic Fibrosis Mice K. GYOMOREY, H. YEGER, R. ROZMAHEL, K.A. GALLEY, E. GARAMI, M. RAMJEESINGH, J.M. ROMMENS, L.-C. TSUI, and C.E. BEAR, *Division of Cell Biology, Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada* (Sponsored by K. Strange)

Background. Cystic Fibrosis (CF) results from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CFTR knock-out mice mimic the heterogeneity in gastrointestinal (GI) disease severity seen in CF patients and hence provide an excellent model for the study of variables that determine the disease phenotype (Rozmahel et al. 1996. *Nat. Genet.* 280:287). As CFTR normally mediates Cl^- secretion in the GI tract, alternative Cl^- channels may compensate for lack of CFTR in the GI tract of CF mice with "mild" disease. Using the Ussing chamber technique, we recently identified a non-CFTR secretory Cl^- conductance in the GI tract of CF mice. Increased activity of this conductance was associated with amelioration of intestinal disease. Functional characterization of this conductance suggests that it is mediated by the ClC-2 Cl^- channel.

Hypothesis. ClC-2 expression in the murine GI tract is consistent with a role in Cl^- secretion. Further, upregulation of ClC-2 expression may account for the improved Cl^- secretory capacity by intestinal epithelia of CF mice with mild disease.

Methods. ClC-2 message and protein expression was compared in the small intestine of CF mice with mild and "severe" disease and normal mice using RT-PCR, Northern analysis, and immunofluorescence.

Results. We found that, overall, there is an increase in ClC-2 message in the intestinal tissue obtained from CF animals when compared with normal mice. Furthermore, ClC-2 mRNA expression in the jejunum of CF mice with mild disease was elevated relative to the expression in the same tissue from CF mice with severe disease. Preliminary immuno-localization studies show that ClC-2 colocalizes with plasma membrane markers in epithelial cells lining the villi and crypts of intestinal mucosa obtained from normal and CF mice.

Conclusion. Our findings indicate that knock out of the CFTR gene in mice results in increased expression of ClC-2 message in the GI tract. In addition, we found that ClC-2 protein localizes to the intestinal epithelial membrane, thus supporting the hypothesis that it contributes to Cl^- secretion. Expression of ClC-2 mRNA is increased in the GI tract of CF mice with mild disease when compared with CF mice with severe disease; this may account for the increased Cl^- secretion in the GI tract of "mildly affected" CF mice. The present work will provide valuable information for determining the potential of ClC-2 as a target for therapeutic intervention to overcome the CF defect in the GI tract. (Supported by CCFF and MRC, K. Gyomorey is a CCFF studentship awardee.)

68. Overexpression of ClC-2 Amino Terminus Reduces Voltage-activated ClC-2 Chloride Currents in *Xenopus* Oocytes EVA A. PASYK, RAHA MOHAMMAD-PANAH, LORA CRUISE, KEVIN GALLEY, and CHRISTINE E. BEAR, *The Hospital for Sick Children, Toronto, Ontario, Canada* (Sponsored by K. Strange)

ClC-2 belongs to a family of voltage-activated chloride channels. A role for the NH_2 terminus of ClC-2 has been implicated in channel gating as its deletion causes constitutive activation. The objective of this study was to assess the role of the NH_2 terminus of ClC-2 as an inhibitory particle by examining the effect of its coexpression with ClC-2 on ClC-2 channel function. ClC-2 cRNA was expressed in *Xenopus* oocytes and chloride channel function was studied using a two-electrode voltage clamp. ClC-2 chloride currents were typically activated by strong hyperpolarizing voltage step of −140 mV (1,582 ± 110 nA, $n = 42$). Coinjection of cRNA (0.16 ng/nl) encoding full length ClC-2 with cRNA (0.32 ng/nl) encoding the NH_2 terminus of ClC-2 (residues: 34–71) produced currents that were reduced in magnitude relative to those recorded in oocytes with ClC-2 alone (424 ± 42 nA, $n = 38$, at −140 mV). This inhibitory effect was specific for ClC-2 NH_2-terminus expression as ClC-2 (0.16 ng/nl) coexpressed with ClC-2 COOH terminus (0.32 ng/nl) caused no inhibition of ClC-2 currents ($n = 9$). In conclusion, the reduction of hyperpolarization-activated currents in oocytes overexpressing the ClC-2 NH_2 terminus supports the hypothesis that this peptide acts as an inhibitory gating particle for ClC-2. (Supported by MRC, Canada, and the Canadian Cystic Fibrosis Foundation.)

69. R Domain Serine 768, a Negative Regulator of CFTR Chloride Channel Gating LASZLO CSANADY, KIM W. CHAN, BENJAMIN B. ANGEL, ANGUS C. NAIRN, and DAVID C. GADSBY, *Laboratory of Cardiac/Membrane Physiology, The Rockefeller University, New York*

Ser768 in the R domain of CFTR appears to be a phosphorylation site that inhibits CFTR channel gating since the dose–response curve for activation of S768A CFTR channels, expressed in *Xenopus* oocytes, by increasing concentrations of IBMX in the presence of forskolin is shifted to lower [IBMX] than that for WT CFTR. Resting unstimulated oocytes display a significant basal CFTR Cl^- conductance ~10% (WT) or ~70% (S768A) of the maximally activatable CFTR Cl^- conductance. Substitution of Ala for S813, one of the most potent stimulatory phosphorylation sites, results in a basal CFTR Cl^- conductance comparable to that of WT CFTR. However, the double mutant S768A/S813A elicits a basal conductance that is intermediate between that of WT and S768A (~30%). These data, taken together with the fact that S768A, S813A, and the double mutant all exhibit maximally activatable (by 1 mM IBMX plus 50 μM forskolin) conductances comparable with that of WT channels, suggest that phosphoserine 768 acts to inhibit phosphorylation of serines within "stimulatory" sites. Analysis of WT and S768A CFTR single-channel currents activated by direct application of purified PKA catalytic subunit to inside-out patches excised from *Xenopus* oocytes confirms that the responsiveness of S768A CFTR channels to PKA is increased relative to WT, as indicated by a much larger P_o value at a given submaximal concentration of PKA. Kinetic analysis shows that at very low [PKA] the opening rate of the mutant is substantially higher than that of the WT, whereas at higher (but still submaximal) concentrations it is the much longer burst duration of the mutant that accounts for its higher P_o. A simple in-