Journal of Bioenergetics and Biomembranes, Vol. 30, No. 2, 1998

The Na-K-Cl Cotransporters

Mark Haas[1] and Bliss Forbush III[2]

Received April 7, 1998

The Na-K-Cl cotransporters are a class of membrane proteins that transport Na, K, and Cl ions into and out of a wide variety of epithelial and nonepithelial cells. The transport process mediated by Na-K-Cl cotransporters is characterized by electroneutrality (almost always with stoichiometry of 1Na:1K:2Cl) and inhibition by the "loop" diuretics bumetanide, benzmetanide, and furosemide. Presently, two distinct Na-K-Cl cotransporter isoforms have been identified by cDNA cloning and expression; genes encoding these two isoforms are located on different chromosomes and their gene products share approximately 60% amino acid sequence identity. The NKCC1 (CCC1, BSC2) isoform is present in a wide variety of tissues; most epithelia containing NKCC1 are secretory epithelia with the Na-K-Cl cotransporter localized to the basolateral membrane. By contrast, NKCC2 (CCC2, BSC1) is found only in the kidney, localized to the apical membrane of the epithelial cells of the thick ascending limb of Henle's loop and of the macula densa. Mutations in the NKCC2 gene result in Bartter's syndrome, an inherited disease characterized by hypokalemic metabolic alkalosis, hypercalciuria, salt wasting, and volume depletion. The two Na-K-Cl cotransporter isoforms are also part of a superfamily of cation-chloride cotransporters, which includes electroneutral K-Cl and Na-Cl cotransporters. Na-K-Cl cotransporter activity is affected by a large variety of hormonal stimuli as well as by changes in cell volume; in many tissues this regulation (particularly of the NKCC1 isoform) occurs through direct phosphorylation/dephosphorylation of the cotransport protein itself though the specific protein kinases involved remain unknown. An important regulator of cotransporter activity in secretory epithelia and other cells as well is intracellular [Cl] ($[Cl]_i$), with a reduction in $[Cl]_i$ being the apparent means by which basolateral Na-K-Cl cotransport activity is increased and thus coordinated with that of stimulated apical Cl channels in actively secreting epithelia.

KEY WORDS: Na-K-Cl cotransporter; K-Cl cotransport; Na-Cl cotransport; bumetanide; furosemide; cell volume regulation; epithelia; kidney.

INTRODUCTION

Cotransport of sodium plus potassium was first described in the early 1970s in red blood cells by Wiley and Cooper.[84] It was not until several years later that the dependence of this cotransport process on chloride was reported,[42] and in 1980 Geck and coworkers[19] provided the first definitive evidence for the electrically neutral cotransport of Na, K, and Cl ions, with stoichiometry of 1Na:1K:2Cl, in Ehrlich ascites tumor cells.

Na-K-Cl cotransporters are now known to be present in numerous, diverse tissues from a wide variety of animal species, where they serve a number of vital physiological functions including ion transport across secretory and absorptive epithelia, maintenance and regulation of cell volume and ion gradients, and possibly modulation of cell growth and development (see Refs. 23, 38, and 62 for recent reviews). In all cells and tissues in which these transporters have been described, the Na-K-Cl cotransporters share several basic properties: (1) The cotransport process is electro-

[1] Department of Pathology, The University of Chicago, Chicago, Illinois 60637.

[2] Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06510.

0145-479X/98/0400-0161$15.00/0

neutral, with a stoichiometry of 1Na:1K:2Cl in nearly all cases, and with an absolute requirement that Na, K, and Cl all be present on the same side of the membrane for net transport to occur. (2) Net transport may occur into or out of the cells, the magnitude and direction of this transport being determined by the sum of the chemical potential gradients of the transported ions, Na, K, and Cl (the Cl term being squared due to the stoichiometry of cotransport). (3) Transport is inhibited by the 5-sulfamoylbenzoic acid "loop" diuretics, which include (in increasing order of potency) furosemide, bumetanide, and benzmetanide. The cotransport mechanism and its inhibition by "loop" diuretics have been reviewed elsewhere.[7,23,46]

During the past several years, two distinct but highly homologous isoforms of Na-K-Cl cotransport proteins have been identified by cDNA cloning, sequencing, and functional expression.[6,17,63,65,88] In addition, cDNAs encoding several additional proteins sharing significant amino acid sequence homology have been identified; the latter proteins include the thiazide-sensitive Na-Cl cotransporters[17,18] and two different isoforms of electrically neutral K-Cl cotransporters.[20,64] These findings establish the existence of a superfamily of related cation-chloride cotransporters, as was previously proposed.[23] The identification of cDNAs encoding the Na-K-Cl cotransporters and the expression of the cotransport proteins has provided researchers with important new means to examine the distribution of the different cotransporter isoforms in various tissues, as well as the regulation of these transporters at a molecular level. This review will focus on certain molecular aspects of the Na-K-Cl cotransporters, including the distribution of Na-K-Cl cotransporter isoforms in different cells and tissues and how this may relate to cotransporter function in these tissues, the primary structure and predicted secondary structure of Na-K-Cl cotransport proteins and other members of the cation-chloride cotransporter superfamily, and the regulation of Na-K-Cl cotransport, particularly by phosphorylation/dephosphorylation of the cotransport protein and by putative interaction with cytoskeletal proteins.

DISTRIBUTION OF Na-K-Cl COTRANSPORTER ISOFORMS

As noted above, two distinct isoforms of the Na-K-Cl cotransporter, NKCC1 and NKCC2, have been identified to date. A cDNA encoding NKCC1 was first cloned from the shark rectal gland,[86] a well-studied secretory epithelium with a high density of Na-K-Cl cotransporters in its basolateral cell membrane as determined from ion transport and "loop" diuretic binding studies.[15] Subsequently, cDNAs encoding NKCC1 have been cloned from other secretory epithelia, including T84 cells, a human colonic epithelial cell line,[65] and cultured inner medullary collecting duct cells (mIMCD-3) from mouse kidney.[6] By northern blot analysis, NKCC1 is present in a wide variety of tissues, including salivary gland, stomach, lung, trachea, and pancreas, all possessing secretory epithelia.[6,65,86] These analyses as well as immunohistchemistry and *in-situ* hybridization also demonstrated the presence of NKCC1 mRNA in nonepithelial tissues such as heart, skeletal muscle, and neurons.[6,65,67,86] Recently, a cDNA encoding the bovine aortic endothelial cell Na-K-Cl cotransporter was cloned and sequenced, and the predicted protein was found to exhibit ~95% amino acid identity with the T84 cell cotransporter sequence,[88] further supporting the conclusion that most if not all nonepithelial Na-K-Cl cotransporters are of the NKCC1 isoform. In intact secretory epithelia, NKCC1 protein is localized specifically to the basolateral cell membrane, as demonstrated by immunofluorescence studies using monoclonal, anti-cotransporter antibodies,[51] as well as [^{3}H]bumetanide binding studies (e.g., Ref. 25). These basolateral cotransporters mediate a net salt influx into the cells, and in doing so act in concert with apical Cl channels and basolateral K channels to produce net salt and fluid secretion in these epithelia (see Regulation of Na-K-Cl Cotransport, below). Panels A and B of Fig. 1 exhibit the cellular distribution of Na-K-Cl cotransport protein in parotid acinar epithelium, a secretory epithelium containing the NKCC1 isoform. One notable exception to the basolateral distribution of NKCC1 is in the rat choroid plexus, where the apical localization of NKCC1 argues against its involvement in ion secretion into cerebrospinal fluid.[67]

In contrast to NKCC1, the NKCC2 isoform of the Na-K-Cl cotransport is localized exclusively to the kidney.[9,17,63] NKCC2 shares ~60% amino acid sequence identity with NKCC1 (Table I; also see below), and these two Na-K-Cl cotransporter isoforms clearly represent different gene products. In the mouse, NKCC1 has been mapped to chromosome 18 and the NKCC2 gene to chromosome 2.[6,70] Immunofluorescence studies with anti-cotransporter antibodies, such as depicted in panels C and D of Fig. 1, have localized

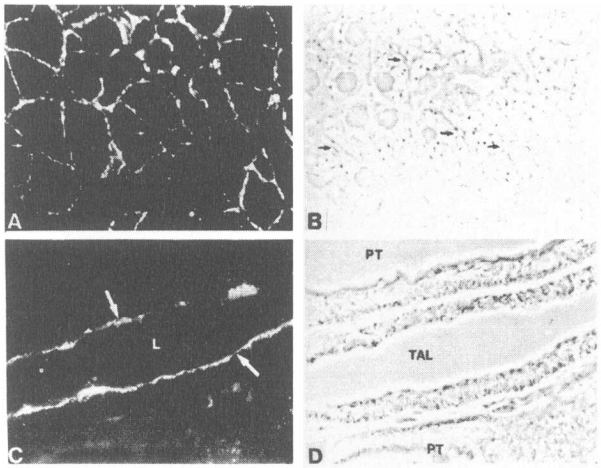

Fig. 1. Immunolocalization of Na-K-Cl cotransporters in cryosections of rabbit parotid gland (NKCC1, panels A and B) and of rabbit renal cortex (NKCC2, panels C and D). Tissue sections were incubated with antibody T4, a mouse monoclonal antibody raised against a fusion protein fragment encompassing the carboxy-terminal 310 amino acids of human colonic NKCC1, followed by fluorescein isothiocyanate-conjugated anti-mouse IgG. Antibody T4 reacts with both NKCC1 and NKCC2 isoforms of Na-K-Cl cotransporters.[51] A: Immunofluorescence microscopy reveals Na-K-Cl cotransporters localized to the basolateral membranes of parotid acinar cells, with no apical labeling (arrows). B: Phase-contrast image of same field shown in A (magnification × 650). The acinar lumens (arrows) are partially collapsed in these unstimulated cells. C: Na-K-Cl cotransporters of renal cortex are localized to the apical membrane (L = lumen) of cells lining the thick ascending limb of Henle's loop (TAL). No staining is detected in the proximal tubule (PT). D: Phase-contrast image of same field shown in C (magnification × 650). Reproduced from Lytle *et al.*[51] with copyright permission from The American Physiological Society.

NKCC2 to the apical membrane of the epithelial cells of the thick ascending limb of Henle's loop [TAL],[39,51] consistent with the known site of action of the 5-sulfamoylbenzoic acid "loop" diuretics.[4] Notably, antibodies specifically directed against NKCC1 failed to label the TAL in immunofluorescence studies.[21] *In-situ* hybridization studies[58] have also demonstrated NKCC2 in the macula densa, where apical Na-K-Cl cotransport may play an important role in the "chloride-sensing" function of these cells.[44]

Payne and Forbush[63] identified three alternatively spliced forms of NKCC2 in the rabbit kidney, with the region of divergence between these NKCC2 variants being an alternatively spliced cassette exon encoding most of the predicted second transmembrane domain and part of the putative intracellular loop connecting transmembrane domains 2 and 3 (63; also see below). Payne and Forbush[63] also found that one form (B) of NKCC2 was expressed only in the cortex, a second (F) only in the medulla, and the third (A) in both cortex and medulla by northern blot analysis. These latter findings were confirmed and extended by *in-situ* hybridization studies of Igarashi *et al.*[30] in the mouse kidney, which localized the B form primarily to the cortical TAL (cTAL), and detected the greatest levels of expression of A and F forms in the outer and inner stripes, respectively, of the outer medulla. In studies of Yang *et al.*[87] using dissected tubule segments from rat kidney and reverse transcription–polymerase chain reaction (RT-PCR), the B form of NKCC1 was detected exclusively in the cortical TAL (cTAL) and macula densa, the F form exclusively in the medullary TAL (mTAL), and the A form in cTAL, macula densa, and mTAL.

This distribution of NKCC2 splice variants may in part be responsible for differences in Na-K-Cl cotransport activity and regulation along the nephron. For example, in the mouse kidney isolated mTAL segments respond to cell shrinkage in the presence of antidiuretic hormone with a volume-regulatory response mediated in part by Na-K-Cl cotransport, whereas isolated cTAL segments fail to exhibit such a response under identical conditions.[80] Interestingly, the region of NKCC2 encoded by the alternatively spliced cassette exon is rich in serine and threonine residues, potential sites for regulatory phosphorylation that are not entirely conserved among the A, B, and F variants of NKCC2.[63] However, the majority of these serine and threonine residues (and all but one of these that is not conserved among A, B, and F forms) lie within putative transmembrane domain 2,[63] and as such may not be accessible to the protein kinase(s) and phosphatase(s) involved in cotransporter regulation (see Regulation of Na-K-Cl Cotransport, below). Two additional splice variants of mouse NKCC2 have been reported by Mount *et al.*[57]; these forms differ at their carboxy-terminal ends and have different consensus phosphorylation sites for cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC). Furthermore, these two variants differ in their response to cyclic AMP (cAMP) when expressed in *Xenopus* oocytes.[57] Both transcripts have been identified in mouse cortex and outer medulla by northern blot and RT-PCR[57], but further characterization of their distribution along the nephron has not yet been reported.

Recently, it was demonstrated by genetic linkage analysis that frameshift and nonconservative missense mutations in the NKCC2 gene result in Bartter's syndrome, a hereditary (autosomal recessive) condition that typically presents in infancy and is characterized

Table I. Properties of Known Cation-Chloride Cotransporter Superfamily Members

NKCC1	
Also known as:	CCC1, BSC2
Prototype:	Shark rectal gland Na-K-Cl cotransporter[86]
Distribution:	Secretory epithelia (basolateral membrane); choroid plexus (apical membrane); nonepithelial tissues
Stoichiometry:	1Na:1K:2Cl
Inhibitors:	Bumetanide > furosemide, insensitive to thiazides
Size of mRNA:	7.0–7.5 kb
Mass of protein:	~130 kDa (deglycosylated)
Homology:	~60% amino acid identity with NKCC2; ~45% amino acid identity with NCC; ~25% amino acid identity with KCC1 and KCC2
NKCC2	
Also known as:	CCC2, BSC1
Prototypes:	Rabbit and rat kidney Na-K-Cl cotransporters[17,63]
Distribution:	Kidney (alternatively spliced variants in cortex and/or medulla)
Stoichiometry:	1Na:1K:2Cl
Inhibitors:	Bumetanide > furosemide; insensitive to thiazides
Size of mRNA:	4.6–5.2 kb
Mass of protein:	120–130 kDa (deglycosylated)
Homology:	~60% amino acid identity with NKCC1; 45–48% amino acid identity with NCC; ~25% amino acid identity with KCC1 and KCC2
NCC	
Also known as:	CCC3, TSC
Prototypes:	Winter flounder urinary bladder and rat kidney Na-Cl cotransporters[17,18]
Distribution:	Teleost urinary bladder, distal tubule of mammalian kidney (apical membrane)
Stoichiometry:	1Na:1Cl, K-independent
Inhibitors:	Metolazone and other thiazide diuretics; insensitive to bumetanide
Size of mRNA:	3.0–4.4 kb
Mass of protein:	~110kDa (deglycosylated)
Homology:	~45% amino acid identity with NKCC1; 45–48% amino acid identity with NKCC2; ~25% amino acid identity with KCC1 and KCC2
KCC1	
Prototypes:	K-Cl cotransporters of many tissues including kidney, brain, erythrocytes[20]
Distribution:	Above, plus heart, lung, liver, muscle, stomach, colon, placenta
Stoichiometry:	1K:1Cl, Na-independent
Inhibitors:	Furosemide > bumetanide; insensitive to thiazides
Size of mRNA:	3.8 kb
Mass of protein:	~120 kDa (deglycosylated)
Homology:	67% amino acid identity with KCC2; ~25% amino acid identity with NKCC1, NKCC2 and NCC
KCC2	
Prototype:	Rat brain K-Cl cotransporter[64]
Distribution:	Neuronal-specific
Stoichiometry:	Not certain; probably 1K:1Cl, Na-independent
Inhibitors:	Not tested
Size of mRNA:	5.6 kb
Mass of protein:	124 kDa (predicted)
Homology:	67% amino acid identity with KCC1; ~25% amino acid identity with NKCC1, NKCC2 and NCC

by renal salt wasting, hypovolemia, hypokalemic metabolic alkalosis, and hypercalciuria.[76,77] Patients with Bartter's syndrome are homozygous for mutations in the NKCC2 gene, and the great majority studied to date have the identical mutation on both chromosomes, due to parental consanguinity.[76] Erythrocyte Na-K-Cl cotransport activity has been found to be normal in patients with Bartter's syndrome,[41] suggesting that NKCC1 is unaffected in this disease. Bartter's syndrome in some ways resembles, but is distinct from Gitelman's syndrome, another autosomal recessive condition leading to hypokalemic metabolic alkalosis; the latter typically presents later in life with hypocalciuria, hypomagnesemia, and without severe volume depletion.[77] Genetic linkage analysis[78] has linked Gitelman's syndrome to nonconservative muta-

tions in the gene for the renal thiazide-sensitive Na-Cl cotransporter, NCC, which shares significant amino acid sequence homology with NKCC1 and NKCC2 (Table I; also see below).

STRUCTURE OF Na-K-Cl COTRANSPORT PROTEINS: THE CATION-CHLORIDE COTRANSPORTER SUPERFAMILY

NKCC1, NKCC2, and NCC are each members of a superfamily of genetically distinct, but structurally and functionally related membrane proteins termed the cation-chloride cotransporter (CCC) superfamily.[23] Properties of the currently known members of this superfamily are summarized in Table I. The CCC superfamily also comprises two distinct K-Cl cotransporter isoforms, the ubiquitous KCC1, and the neuronal-specific KCC2.[20,64] Each member of the CCC superfamily shares varying degrees of amino acid sequence identity with the other superfamily members, ranging from 67% between the two KCC isoforms to ~25% between the KCC isoforms and the other CCCs. Evidence to date suggests that a single subunit is sufficient for cotransporter function (e.g., Ref. 86), though this has yet to be definitively addressed. These proteins also share a common predicted secondary structure. As illustrated in Fig. 2 (which shows the predicted secondary structure for NKCC1 from shark rectal gland), this secondary structure is characterized by 12 predicted transmembrane domains, and hydrophilic, putatively intracellular amino- and carboxy-terminal domains. As shown for NKCC1 in Fig. 2, each member of the CCC superfamily also possesses one or more potential N-linked glycosylation sites on predicted extracellular loops of the protein, and several consensus phosphorylation sites for known protein kinases within the predicted amino- and carboxy-terminal domains.[6,17,18,20,63,64,65,86,88] The presence of potential extracellular glycosylation sites is consistent with the decrease in apparent molecular mass on SDS-polyacrylamide gels that has been demonstrated for several of these proteins in their physiological plasma membrane environment following treatment of the membranes with an aminoglycosidase.[17,20,65] Site-directed mutagenesis performed on the rat renal Na-Cl cotransporter (NCC; Table I) demonstrated that Asn 404, a potential N-linked glycosylation site that is highly conserved among CCC superfamily members (and corresponds to the glycosylation site closest to the amino-terminus among those indicated in the extracellular

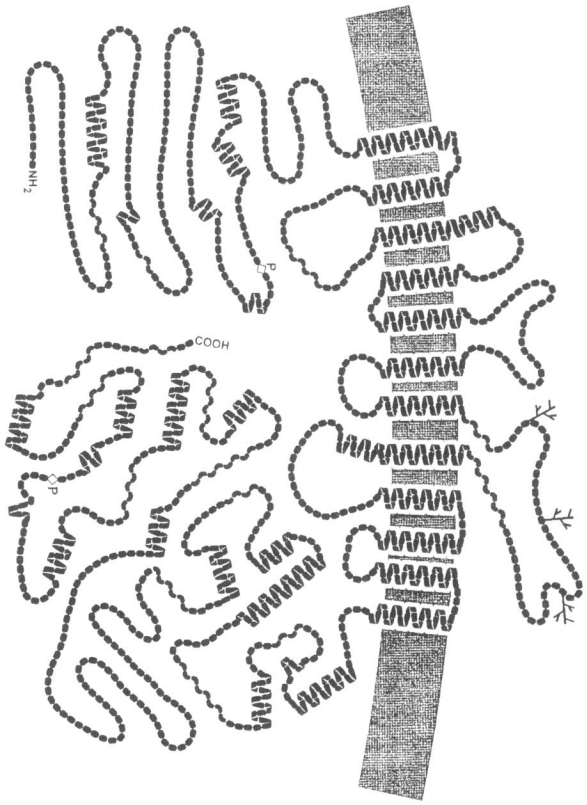

Fig. 2. Model of the shark rectal gland Na-K-Cl cotransport protein (NKCC1), based on its cDNA sequence and hydropathy and secondary structural analyses. Each link symbolizes a single amino acid residue, of which there are a total of 1191. Amino- and carboxy-termini are indicated by "NH_2" and "COOH," respectively. Branched lines indicate potential glycosylation sites between putative transmembrane segments 7 and 8. Biochemically identified phosphorylated threonine residues (positions 189 and 1114; Refs. 49, 86) are indicated by open diamonds with an adjacent "P." The proposed secondary structure of this protein with 12 transmembrane helices and large intracellular amino- and carboxy-terminal domains is shared by other members of the cation-chloride cotransporter (CCC) superfamily that have been cloned and sequenced (Table I.)

loop between transmembrane domains 7 and 8 in Fig. 2), is in fact glycosylated when NCC protein is expressed in *Xenopus* oocytes.[68] Both glycosylated and unglycosylated forms of NCC could be expressed in the oocyte plasma membrane, though kinetic analysis of transport studies suggested that glycosylation may augment cell surface expression of NCC.[68]

The greatest degree of sequence homology among members of the CCC superfamily is in the predicted transmembrane domains, and in putative intracellular loops connecting these domains, particularly the large loop connecting the second and third transmembrane domains.[6,17,18,20,63,64,65,86,88] Regions of significant

homology are also noted within the carboxy-terminal domain, and to a lesser extent within the amino-terminal domain. Regions exhibiting a high degree of homology among different CCC family members also tend to exhibit the greatest degree of cross-species homology (e.g., between human and shark NKCC1; Ref. 65). By contrast, considerable divergence between the primary structures of CCC superfamily members is noted in the putative extracellular loops connecting transmembrane domains, which are likely to represent sites involved in the binding of ions and inhibitors. These differences in primary structure are thus likely to underlie the marked differences in ion and inhibitor selectivity and affinity between CCC superfamily members. Studies employing site-directed mutagenesis are just beginning to provide information as to specific regions of cotransport proteins that may be involved in the binding of specific ions and/or inhibitors. Isenring and coworkers[31,32] recently studied the ion and bumetanide affinities of several different chimeras of human colonic NKCC1 and shark rectal gland NKCC1; these two Na-K-Cl cotransporters exhibit rather different affinities for extracellular Na, K, Cl, and bumetanide. They found that changes in the region of predicted transmembrane domain 2 affected cation but not chloride affinity, and that a 145-amino acid sequence spanning from predicted transmembrane domains 4–7 determined chloride affinity. These workers[32] also concluded that bumetanide binding was likely to be influenced primarily by regions of the molecule beyond the seventh predicted transmembrane domain, consistent with recent evidence that bumetanide binding to the rabbit parotid Na-K-Cl cotransporter, though strongly influenced by extracellular [Cl], does not appear to occur at a chloride transport site.[56]

It is likely that in the upcoming years additional members of the CCC superfamily will be added to those listed in Table I. Considerable homology is known to exist between NKCC and KCC isoforms and sequences present in cyanobacteria and *Caenorhabditis elegans*.[43,63,65,86] It also remains to be determined what the relationship may be between the molecules listed in Table I and several described cation-chloride cotransporters with notably different properties, including electrogenic K-Cl cotransporters,[45] bumetanide-sensitive, K-independent Na-Cl cotransporters in mouse and rabbit mTAL,[13,79] and the Na-K-Cl cotransporter of squid giant axon, which has a reported stoichiometry of 2Na:1K:3Cl.[72]

REGULATION OF Na-K-Cl COTRANSPORT

The regulation of Na-K-Cl cotransport is extremely diverse, and varies greatly among different cells and tissues (e.g., see Table 2 of Ref. 22). In addition to various hormones, second messengers, and other physiological perturbations (e.g., changes in cell volume) that influence the activity of the cotransporter protein in the plasma membrane, Na-K-Cl cotransport may also be regulated at the level of mRNA transcription. This section of the review will examine several aspects of Na-K-Cl cotransport regulation but will focus primarily on two: regulatory phosphorylation/dephosphorylation of the Na-K-Cl cotransport protein, and regulation of cotransport activity by cytoskeletal proteins that may interact with the cotransporter. It is hypothesized that these represent two highly distinct mechanisms for Na-K-Cl cotransport regulation: phosphorylation puts the cotransport protein in an "active" conformation that readily binds Na, K, and Cl ions (as well as "loop" diuretics), whereas interaction with specific cytoskeletal elements is required for effective ion translocation once the appropriate ion binding sites on the protein are fully occupied.

Regulation of Na-K-Cl Cotransporter Gene Expression

This area of study is essentially in its infancy, with studies reported to date limited to the kidney and to the NKCC2 isoform. In two separate studies, chronic furosemide administration to rats was found to increase levels of NKCC2 mRNA and protein expression.[9,55] Chronic oral saline loading also increased NKCC2 protein expression in the rat outer medulla, but a five-day infusion of vasopressin or water deprivation for 48 hours did not.[9] Thirsting was also found to have no effect on cortical expression of NKCC2 protein,[9] though in a separate study dehydration of rats was found to increase Na-K-Cl cotransporter mRNA levels in microdissected distal convoluted tubules, but not in the mTAL.[33]

Regulation by Phosphorylation/Dephosphorylation

It has been known for over a decade that the activation of Na-K-Cl cotransport in various cell types by a variety of hormonal stimuli and by cell shrinkage

is blocked by different protein kinase inhibitors, and that several different inhibitors of protein phosphatases activate Na-K-Cl cotransport in the absence of other stimuli (see Refs. 23 and 62 for reviews). Lytle and Forbush[49] first demonstrated direct phosphorylation of the Na-K-Cl cotransport protein itself, in shark rectal gland tubules exposed to cAMP-dependent stimuli of salt secretion in this epithelium (vasoactive intestinal peptide [VIP], forskolin) or to hypertonic media. Furthermore, it was shown that the level of cotransport protein phosphorylation was closely correlated with the level of specific [^{3}H]benzmetanide binding to intact rectal gland tubules,[49] in several tissues including the shark rectal gland it has been shown that levels of Na-K-Cl cotransport activity and specific [^{3}H]benzmetanide or [^{3}H]bumetanide binding increase or decrease in approximate proportion to one another in response to various physiological stimuli or inhibitors, respectively, of Na-K-Cl cotransport (e.g., Refs. 15, 16, 24, 25, 36, 59, 61, and 66). Direct phosphorylation of the Na-K-Cl cotransport protein in response to hormonal cotransport stimuli, their putative second messengers, and cell shrinkage has now been demonstrated in a number of different tissues containing the NKCC1 isoform, including other secretory epithelia[27,81,82] and endothelial cells[40,60]. In the shark rectal gland, phosphorylation of NKCC1 occurs on serine and threonine, but not tyrosine residues, and two phosphothreonine residues have been identified within the amino-terminal and carboxy-terminal domains of the protein, respectively (Refs 49 and 86; also see Fig. 2). Still, the specific protein kinases responsible for cotransport protein phosphorylation are unknown. Lytle and Forbush[49] hypothesized that cotransport activation by forskolin in the shark rectal gland is not due to phosphorylation of the cotransport protein by PKA, and shark NKCC1 indeed lacks consensus PKA phosphorylation sites.[86] Studies in intact tubules isolated from shark rectal gland provide strong evidence that stimuation of basolateral Na-K-Cl cotransport in this tissue by VIP and forskolin is entirely secondary to cAMP-dependent activation of apical chloride channels,[50] supporting the above hypothesis. By contrast, in canine and human tracheal epithelium, evidence for direct activation of Na-K-Cl cotransport for PKA has been presented,[23,28] though this clearly cannot account for the full extent of cotransport activation by cAMP-dependent secretagogues, and cannot account at all for cotransport activation by cell shrinkage and cAMP-independent secretagogues such as apical UTP.[23,28]

Although the specific protein kinases responsible for cotransporter phosphorylation and activation remain unknown, considerable evidence now exists that: (1) in secretory epithelia stimulated by hormonal secretagogues, the activation of basolateral Na-K-Cl cotransport that occurs results from a true up-regulation of the cotransporter related to cotransport protein phosphorylation, and is not a thermodynamic effect releated to changes in ion gradients (reviewed in Ref. 23); (2) cotransporter phosphorylation and activation is at least in part secondary to event(s) related to hormonal activation of apical membrane chloride channels[26–28]; and (3) a likely candidate for the intracellular mediator leading to cotransport protein phosphorylation is a reduction in intracellular chloride concentration, $[Cl]_i$.[27,50] Evidence that a reduction in $[Cl]_i$, in the absence of hormonal stimuli or a concomitant cell shrinkage, can activate Na-K-Cl cotransport was first presented by Breitwieser *et al.*[3] in the internally dialyzed squid giant axon. This finding has been confirmed and extended in secretory epithelial cells of the shark rectal gland[50] and canine trachea[26–28]; in both of these cell types lowering $[Cl]_i$ in the absence of secretagogue or concomitant cell shrinkage results in activation of basolateral Na-K-Cl cotransport, and approximately proportional increases in specific "loop" diuretic binding and NKCC1 phosphorylation. Figures 3 and 4 illustrate an example of this in confluent primary cultures of tracheal epithelial cells treated with apical nystatin to increase apical Cl permeability to a degree where the rate of basolateral-to-apical ^{36}Cl flux is determined by the rate of basolateral ^{36}Cl influx via the Na-K-Cl cotransporter.[26] In Figure 3, basolateral-to-apical ^{36}Cl flux was first measured for 10 min at 124 or 66 mM apical [Cl] ($[Cl]_a$), after which time $[Cl]_a$ was decreased from 124 to 66 mM, or from 66 to 32 mM. The experiments were done in the absence of secretagogue, and cell shrinkage is avoided by substituting equimolar amounts of nitrate for chloride, both anions being highly permeant across the nystatin-treated apical membrane. Decreasing $[Cl]_a$, and thus $[Cl]_i$, increased bumetanide-sensitive, basolateral-to-apical ^{36}Cl flux as much as threefold within a 20-min period (Fig. 3). In the experiment shown in Fig. 4, similar cultures of tracheal cells were loaded with ^{32}P, treated with apical nystatin, and then incubated for 20 min with apical media of varying [Cl] between 124 and 32 mM (nitrate substitution; Ref. 27). NKCC1, which has a molecular mass of ~170 kDa in these cells, was then isolated by immunoprecipitation using a monoclonal antibody directed against NKCC1 of

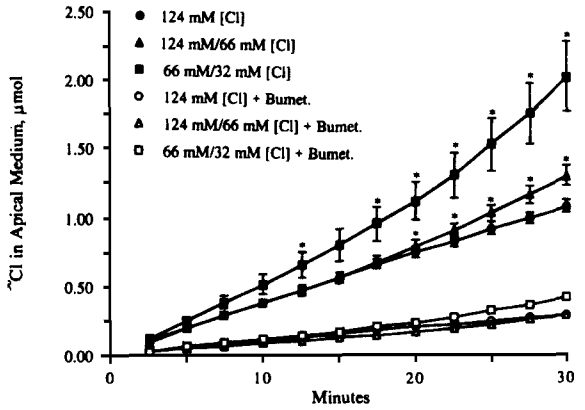

Fig. 3. Effect of reducing apical [Cl] ($[Cl]_a$) on basolateral-to-apical ^{36}Cl flux across confluent primary cultures of dog tracheal epithelial cells, in the presence of apical nystatin. Cultures grown on Transwell-COL supports (Costar) were preincubated for 10 min in the presence of apical nystatin (final concentration 350 units/ml; added from a stock solution in dimethyl sulfoxide, final concentration 0.175% v/v) and 124 mM or 66 mM $[Cl]_a$ (NO_3 replacing Cl); the basolateral medium contained 124 mM [Cl]. Bumetanide (50 µM) was included in the basolateral medium during preincubation for those incubations which contained bumetanide during flux determinations. At the start of flux incubations, $[Cl]_a$ was the same as present during preincubations; the basolateral medium contained 124 mM [Cl] (with or without 50 µM bumetanide) throughout all flux incubations. Nystatin was present apically during the entire 30-min flux incubation. Data points represent the cumulative amount of ^{36}Cl collected from the apical medium, which was collected and replaced with fresh medium every 2.5 min. Where indicated, after collection of the apical medium at 10 min, the apical medium was changed from 124 mM to 66 mM [Cl] (124 mM/66 mM [Cl]) or from 66 mM to 32 mM [Cl] (66 mM/32 mM [Cl]); this lower level of $[Cl]_a$ was then maintained over the ensuing 20 min. Data plotted on the ordinate represent mean values of three determinations (± S.D.; error bars, for incubations without bumetanide) or of two determinations (incubations with bumetanide), using cells cultured from two different dog tracheas. The lines linking the data points were drawn by eye. *The amount of ^{36}Cl accumulated in the apical medium during the specific 2.5-min period ending at the time indicated on the abscissa is significantly different ($p < 0.05$ by paired t test) from that accumulated during the same time period with $[Cl]_a$ = 124 mM. Reproduced from Ref. 26 with copyright permission from The American Physiological Society.

human colonic epithelial cells. The autoradiogram in Fig. 4 shows a progressive increase in NKCC1 phosphorylation in cells exposed to decreasing levels of $[Cl]_a$, and thus having decreasing levels of $[Cl]_i$. Cells incubated for 20 min with 32 mM $[Cl]_a$ had ~3-fold higher levels of NKCC1 phosphorylation than cells incubated under identical conditions except that $[Cl]_a$ was 124 mM; this increase in phosphorylation is similar to the increase in Na-K-Cl cotransport activity noted in similarly incubated cells (see Ref. 27; also see above).

Evidence that reducing $[Cl]_i$ stimulates Na-K-Cl cotransport has also been presented in HEK-293 cells,[86] endothelial cells,[61] and Ehrlich ascites tumor cells.[47] In rat parotid acini, Robertson and Foskett[71] showed that carbachol-stimulated net salt and fluid secretion was accompanied by a rise in intracellular ionized calcium and a fall in $[Cl]_i$ and in cell volume, and that the changes in calcium and in $[Cl]_i$, but not the cell shrinkage, were required for the increase in basolateral sodium influx that is an essential component of net secretion. In this tissue, basolateral sodium influx is mediated by both Na/H exchange and Na-K-Cl cotransport; if the results of Robertson and Foskett[71] apply to Na-K-Cl cotransport this would extend the above-cited studies in other secretory epithelia by confirming that a fall in $[Cl]_i$, and not cell shrinkage [which occurs at least transiently in response to secretagogues in multiple secretory epithelia (e.g., Ref. 75) and as noted above also results in direct phosphorylation of NKCC1], is the primary signal coupling apical Cl channel and basolateral Na-K-Cl cotransport activities during secretion. An alternative mechanism proposed for the upregulation of basolateral Na-K-Cl cotransport in carbachol-stimulated rat parotid acini is that the generation of a product of the cytochrome P450 pathway of arachidonate metabolism is the primary signal for this event, rather than a decrease in $[Cl]_i$.[12] Cytochrome P450 arachidonate metabolites have been shown to regulate Na-K-Cl cotransport activity in rabbit mTAL cells, though in the latter the effect of these metabolites on cotransport is inhibitory rather than stimulatory.[10,11]

The concepts of cell volume-sensitive and $[Cl]_i$-sensitive protein kinases have drawn significant interest over the past several years. A protein kinase (V-kinase) that is deactivated by cell swelling has been postulated to be involved in the regulation of the erythrocyte K-Cl cotransporter (KCC1).[8,34] V-kinase activity is proposed to inhibit K-Cl cotransport, and staurosporine, a nonspecific inhibitor of serine-threonine protein kinases, has been found to activate K-Cl cotransport in erythrocytes.[2,14] However, evidence suggests that factors in addition to phosphorylation/dephosphorylation regulate erythrocyte K-Cl cotransport activity,[73] and (unlike the case with NKCC1) direct, regulatory phosphorylation of KCC1 has not yet been demonstrated. The identity of the V-kinase also remains unknown; it is not PKA or PKC though in swollen avian erythrocytes activation of PKA does inhibit K-Cl cotransport while simultaneously stimulating Na-K-Cl cotransport.[29] K-Cl cotransport in

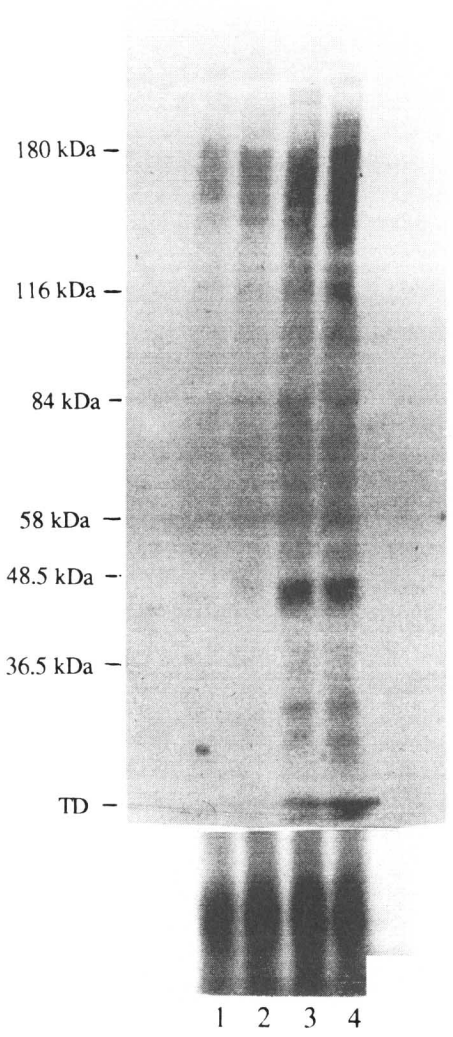

Fig. 4. Phosphorylation of the dog tracheal epithelial Na-K-Cl cotransport protein in response to increasing degrees of reduced $[Cl]_i$ in the absence of secretagogues. ^{32}P-labeled Transwell cultures (six per sample) similar to those used in the experiments described in Fig. 3 were incubated for 20 min with 350 units/ml apical nystatin and 124 mM apical [Cl], followed by an additional 20 min of incubation in the continued presence of apical nystatin and 124 mM apical [Cl] (control; lane 1), 66 mM apical [Cl] (lane 2), 49 mM apical [Cl] (lane 3), or 32 mM apical [Cl] (lane 4). Nitrate substituted isosmotically for Cl; under these conditions apical [Cl] can be reduced from 124 to 32 mM without concurrent cell shrinkage.[26] After the incubations, plasma membranes were isolated from each sample by sonication followed by centrifugation before SDS solubilization of proteins. The SDS-solubilized membrane proteins were then subjected to immunoprecipitation using antibody T4 (see Fig. 1 legend). Immunoprecipitated material was run on a SDS-polyacrylamide gel and proteins were transferred to Immobilon for autoradiography and subsequent western blotting with antibody T4. The upper portion of the figure shows the autoradiogram; the lower portion shows the ~170 kDa region of the western blot subsequently performed on the same transfer. In this experiment, relative amounts of ^{32}P incorporation (per unit protein) into the ~170 kDa Na-K-Cl cotransport protein are: lane 1, 1.0; lane 2, 1.4; lane 3, 2.3; lane 4, 3.0. Reproduced from Ref. 27 with copyright permission from The American Society for Biochemistry and Molecular Biology.

sheep erythrocytes also appears to be influenced by tyrosine kinase activity, though in contrast to staurosporine the tyrosine kinase inhibitor genistein has been found to inhibit K-Cl cotransport in these cells.[14]

Two kinase activities that are progressively inhibited by raising [Cl] in the range of 50–150 mM have been described in plasma membranes from human nasal epithelial cells, though the molecular masses of the substrates for these latter kinases were found to be 37 and 45 kDa, clearly different from NKCC1.[83] Kinetic studies of Na-K-Cl cotransport in squid axon (1) argue against a protein phosphatase being the primary site of $[Cl]_i$-dependent cotransport regulation, but do not exclude a protein kinase in this regard. Interestingly, recent data of Lytle[48] suggest that reducing $[Cl]_i$ and cell shrinkage may each ultimately activate the same protein kinase that phosphorylates NKCC1. Lytle[48] found that proteolytic peptide maps derived from the Na-K-Cl cotransport protein of avian erythrocytes that underwent hypertonic cell shrinkage, reduction of $[Cl]_i$ at constant cell volume, or exposure to the protein phosphatase inhibitior calyculin A all exhibited qualitatively indistinguishable patterns of phosphorylated peptides.

Role of the Cytoskeleton in Na-K-Cl Cotransporter Regulation

Present evidence suggests that a number of cytoskeletal proteins may regulate the activity of both NKCC1 and NKCC2 isoforms of Na-K-Cl cotransporters, most notable among these being filamentous (F-) actin. Studies originally performed in the T84 intestinal epithelial cell line,[52, 74] and subsequently in a mouse mTAL cell line,[85] demonstrated that when these cells are stimulated with agents that increase cellular cAMP levels, a redistribution of F-actin within the cells accompanies the increase in transepithelial ion transport that occurs. When the cells are pretreated with the actin stabilizing agents phalloidin or phallicidin, not only is the redistribution of F-actin prevented, but the cAMP-stimulated increase in transepithelial ion transport (measured as short-circuit current, I_{SC}) is also

largely inhibited. In T84 cells and mouse mTAL cells, as well as in the polarized Cl.19A subclone of the HT29 intestinal epithelial cell line, this inhibitory effect of phalloidin on I_{SC} is due entirely to inhibition of Na-K-Cl cotransport, with no significant effect on other individual transporters that contribute to I_{SC}, namely Cl and K channels, and the Na-K pump.[52,54,85] In the Cl.19A cells, the increase in I_{SC} and in basolateral Na-K-Cl cotransport activity seen in response to cAMP was accompanied by an increase in [^{3}H]bumetanide binding, consistent with findings in other secretory epithelia (e.g., Refs. 15 and 25). By contrast, while phalloidin loading of these cells inhibited the cAMP-induced increases in I_{SC} and in basolateral, bumetanide-sensitive tracer K (^{86}Rb) influx, this treatment did not inhibit the increase in [^{3}H]bumetanide binding promoted by cAMP in these cells.[54] As discussed above, both hormonal stimuli of Na-K-Cl cotransport as well as cell shrinkage promote an increase in bumetanide-sensitive ion transport that is paralleled by increases in specific [^{3}H]bumetanide (or [^{3}H]benzmetanide) binding and in phosphorylation of the Na-K-Cl cotransport protein in a number of cell types, including secretory epithelia[26,27,49] and endothelial cells.[40,60] Thus, the findings in cAMP-stimulated Cl.19A cells [54] suggest that the inhibition of Na-K-Cl cotransport by phalloidin treatment occurs distal, or subsequent, to cotransport protein phosphorylation, perhaps at the level of ion translocation. As noted below, this appears to be a property that is also observed in another apparent instance of Na-K-Cl cotransport regulation at the level of the cytoskeleton.

Recent studies in T84 cells[53] as well as earlier studies in Ehrlich ascites cells[35] demonstrated Na-K-Cl cotransport activation by the cytochalasins, which disrupt F-actin microfilaments. While disruption of actin filament organization may increase the concentration (in the vicinity of the plasma membrane) of so-called "short" actin filaments that have been postulated to be important in the activation of other ion transporters (e.g., epithelial Na channels; Ref. 69), the mechanism of cytochalasin activation of Na-K-Cl cotransport, and whether this is or is not related to cotransport protein phosphorylation, remains unclear. Recent data[5] suggest that the effect of F-actin on Na-K-Cl cotransport may be related to, or perhaps mediated by, two distinct proteins of molecular mass 160 and 130 kDa that coprecipitate with the Na-K-Cl cotransport protein of T84 cells when the latter protein is isolated by immunoprecipitation with a monoclonal antibody directed against NKCC1. Interestingly, when T84 cells were stimulated with cAMP-dependent agonists, levels of these 160 and 130 kDa proteins (but not of the Na-K-Cl cotransport protein itself) detectable by cell surface biotinylation increased sixfold, an increase that was markedly attenuated by pretreating the cells with phalloidin.[5]

Cytoskeletal proteins other than F-actin may also be involved in Na-K-Cl cotransport regulation. In aortic and umbilical vein endothelial cells, cell shrinkage concurrently increased Na-K-Cl cotransport activity and the phosphorylation of myosin light chains (MLC). Subsequent reswelling of the cells reversed the increases in both cotransport activity and MLC phosphorylation; the time courses of the observed changes in both of these responses were generally similar following both initial cell shrinkage and reswelling.[40] Furthermore, both cotransporter activation and MLC phosphorylation in response to cell shrinakge were inhibited by ML-7, an inhibitor of MLC kinase, with similar dose responses.[40] These findings suggested that either: (1) MLC kinase phosphorylates and activates the Na-K-Cl cotransporter of shrunken endothelial cells, or (2) that the activation of endothelial cell Na-K-Cl cotransport by cell shrinkage somehow involves MLC, and that phosphorylation of MLC is necessary for this process to occur. Data of Klein and O'Neill[40] support the latter hypothesis, as the phosphorylation of the endothelial Na-K-Cl cotransporter, which is increased by cell shrinkage,[40,60] was not affected by ML-7 under hypertonic conditions. This finding is also consistent with the hypothesis that regulation of Na-K-Cl cotransport at the level of the cytoskeleton occurs distal to cotransport protein phosphorylation.

Urea, which may alter protein–protein interactions as well as tertiary protein structure even at subdenaturing concentrations, has been found to inhibit Na-K-Cl cotransport in a mouse mTAL cell line.[37] While this effect of urea could possibly be related to cotransporter-cytoskeletal interactions, it was found that this effect could be reversed by removing the urea, and could be prevented by pretreating the cells with the protein phosphatase inhibitor okadaic acid (OA). As such, a more likely explanation for this inhibitory effect of urea on Na-K-Cl cotransport activity would be that urea treatment inhibits a constituitively active protein kinase that activates the cotransporter, or that urea activates an OA-sensitive protein phosphatase which deactivates the cotransporter.[37]

FUTURE DIRECTIONS

The Na-K-Cl cotransporters have historically been relatively difficult to study; their electrically neutral transport mechanism does not allow for studies using patch-clamp techniques and efforts to examine cotransporter function or regulation in various subcellular preparations (e.g., membrane vesicles) or in reconstituted systems have met with only very limited success. Recent successes in cDNA cloning and functional expression of Na-K-Cl cotransporter isoforms as well as of Na-Cl and K-Cl cotransporters, and in the development of specific antibodies directed against these transport proteins, have already begun to rapidly accelerate our understanding of the function and regulation of these transporters in different tissues, a process that should continue in upcoming years. By employing recombinant DNA methodologies to prepare functional chimeras of different CCC superfamily members with different ion and inhibitor affinities and specificities, specific portions of CCC molecules responsible for Na, K, Cl, and inhibitor binding can be deduced. Such studies, as well as experiments employing site-directed mutagenesis of individual CCC superfamily members, should aid us in localizing specific phosphorylation sites involved in cotransporter regulation by hormones, cell shrinkage, and changes in $[Cl]_i$, as well as possible sites of interaction with cytoskeletal proteins. The expected identification of additional CCC superfamily members, with distinct structural and functional similarities and differences from those already known, can only serve to further increase our understanding of and general interest in this unique class of transport proteins.

ACKNOWLEDGMENTS

Original scientific work of the authors was supported by NIH/NIDDK grants DK-17433 and DK-47661 (to B.F. III) and DK-43967 (to M.H.). M.H. is the recipient of an Established Investigatorship award from the American Heart Association. We thank Dr. Chris Lytle for many helpful discussions.

REFERENCES

1. Altamirano, A. A., Breitwieser, G. E., and Russell, J. M. (1995). *Am. J. Physiol.* **269**, C878–C883.
2. Bize, I., and Dunham, P. B. (1994). *Am. J. Physiol.* **266**, C759–C770.
3. Breitwieser, G. E., Altamirano, A. A., and Russell, J. M. (1990). *Am. J. Physiol.* **258**, C749–C753.
4. Burg, M., Stoner, L., Cardinal, J., and Green, N. (1973). *Am. J. Physiol.* **225**, 119–124.
5. D'Andrea, L., Lytle, C., Matthews, J. B., Hofman, P., Forbush, B., III, and Madara, J. L. (1996). *J. Biol. Chem.* **271**, 28969–28976.
6. Delpire, E., Rauchman, M. I., Beier, D. R., Hebert, S. C., and Gullans, S. R. (1994). *J. Biol. Chem.* **269**, 25677–25683.
7. Duhm, J. (1987). *J. Membr. Biol.* **98**, 15–32.
8. Dunham, P. B., Klimczak, J., and Logue, P. J. (1993). *J. Gen. Physiol.* **101**, 733–766.
9. Ecelbarger, C. A., Terris, J., Hoyer, J. R., Nielsen, S., Wade, J. B., and Knepper, M. A. (1996). *Am. J. Physiol.* **271**, F619–F628.
10. Escalante, B., Erlij, D., Falck, J. R., and McGiff, J. C. (1991). *Science* **251**, 799–802.
11. Escalante, B., Erlij, D., Falck, J. R., and McGiff, J. C. (1994). *Am. J. Physiol.* **266**, C1775–C1782.
12. Evans, R.L., and Turner, R.J. (1997). *J. Physiol. (London).* **499**, 351–359.
13. Eveloff, J. L., and Calamia, J. (1986). *Am. J. Physiol.* **250**, F176–F180.
14. Flatman, P. W., Adragna, N. C., and Lauf, P. K. (1996). *Am. J. Physiol.* **271**, C255–C263.
15. Forbush, B., III, Haas, M., and Lytle, C. (1992). *Am. J. Physiol.* **262**, C1000–C1008.
16. Franklin, C. C., Turner, J. T., and Kim, H. D. (1989). *J. Biol. Chem.* **264**, 6667–6673.
17. Gamba, G., Miyanoshita, A., Lombardi, M., Lytton, J., Lee, W.-S., Hediger, M. A., and Hebert, S. C. (1994). *J. Biol. Chem.* **269**, 17713–17722.
18. Gamba, G., Saltzberg, S. N., Lombardi, M., Miyanoshita, A., Lytton, J., Hediger, M. A., Brenner, B. M., and Hebert, S. C. (1993). *Proc. Natl. Acad. Sci. USA* **90**, 2749–2753.
19. Geck, P., Pietrzyk, C., Burckhardt, B.-C., Pfeiffer, B., and Heinz, E. (1980). *Biochim. Biophys. Acta* **600**, 432–447.
20. Gillen, C. M., Brill, S., Payne, J. A., and Forbush, B., III (1996). *J. Biol. Chem.* **271**, 16237–16244.
21. Ginns, S. M., Knepper, M. A., Ecelbarger, C. A., Terris, J., He, X., Coleman, R. A., and Wade, J. B. (1996). *J. Am. Soc. Nephrol.* **7**, 2533–2542.
22. Haas, M. (1989). *Annu. Rev. Physiol.* **51**, 443–457.
23. Haas, M. (1994). *Am. J. Physiol.* **267**, C869–C885.
24. Haas, M., and Forbush, B., III (1986). *J. Biol. Chem.* **261**, 8434–8441.
25. Haas, M., Johnson, L. G., and Boucher, R. C. (1990). *Am. J. Physiol.* **259**, C557–C569.
26. Haas, M., and McBrayer, D. G. (1994). *Am. J. Physiol.* **266**, C1440–C1452.
27. Haas, M., McBrayer, D., and Lytle, C. (1995). *J. Biol. Chem.* **270**, 28955–28961.
28. Haas, M., McBrayer, D. G., and Yankaskas, J. R. (1993). *Am. J. Physiol.* **264**, C189–C200.
29. Haas, M., and McManus, T. J. (1985). *J. Gen. Physiol.* **85**, 649–667.
30. Igarashi, P., Vanden Huevel, G. B., Payne, J. A., and Forbush, B., III (1995). *Am. J. Physiol.* **269**, F405–F418.
31. Isenring, P., Behnke, R., and Forbush, B., III (1995). *J. Am. Soc. Nephrol.* **6**, 341 (abstract).
32. Isenring, P., and Forbush, B. III (1997). *J. Biol. Chem.* **272**, 24556–24562.
33. Itoh, K., Nonoguchi, H., Kitamura, K., Wakamatu, S., Yamakawa, K., and Tomita, K. (1996). *J. Am. Soc. Nephrol.* **7**, 1282 (abstract).
34. Jennings, M. L., and Al-Rohil, N. (1990). *J. Gen. Physiol.* **95**, 1021–1040.

35. Jessen, F., and Hoffmann, E. K. (1992). *Biochim. Biophys. Acta* **1110**, 199–201.
36. Kaji, D. M. (1993). *Biochim. Biophys. Acta* **1152**, 289–299.
37. Kaji, D. M., Diaz, J., and Parker, J. C. (1997). *Am. J. Physiol.* **272**, C615–C621.
38. Kaplan, M. R., Mount, D. B., Delpire, E., Gamba, G., and Hebert, S. C. (1996). *Annu. Rev. Physiol.* **58**, 649–668.
39. Kaplan, M. R., Plotkin, M. D., Lee, W. -S., Xu, Z.-C., Lytton, J., and Hebert, S. C. (1996). *Kidney Int.* **49**, 40–47.
40. Klein, J. D., and O'Neill, W. C. (1995). *Am. J. Physiol.* **269**, C1524–C1531.
41. Korff, J. M., Siebens, A. W., and Gill, J. R., Jr. (1984). *J. Clin. Invest.* **74**, 1724–1729.
42. Kregenow, F. M., and Caryk, T. (1979). *Physiologist* **22**, 73 (abstract).
43. Kumar, S., Warner, F., Logue, P., Dunham, P. B., and Holtzman, E. J. (1996). *J. Am. Soc. Nephrol.* **7** 1283 (abstract).
44. Lapointe, J.-Y., Laamarti, A., Hurst, A. M., Fowler, B. C., and Bell, P. D. (1995). *Kidney Int.* **47**, 752–757.
45. Larson, M., and Spring, K. R. (1984). *J. Membr. Biol.* **81**, 219–232.
46. Lauf, P. K., McManus, T. J., Haas, M., Forbush, B., III, Duhm, J., Flatman, P. W., Saier, M. H., Jr., Russell, J. M. (1987). *Fed. Proc.* **46**, 2377–2394.
47. Levinson, C. (1990). *Biochim. Biophys. Acta* **1021**, 1–8.
48. Lytle, C. Y. (1997). *J. Biol. Chem.* **272**, 15069–15077.
49. Lytle, C., and Forbush, B., III (1992). *J. Biol. Chem.* **267**, 25438–25443.
50. Lytle, C., and Forbush, B., III (1996). *Am. J. Physiol.* **270**, C437–C448.
51. Lytle, C., Xu, J.-C., Biemesderfer, D., and Forbush, B., III (1995). *Am. J. Physiol.* **269**, C1496–C1505.
52. Matthews, J. B., Awtrey, C. S., and Madara, J. L. (1992). *J. Clin. Invest.* **90**, 1608–1613.
53. Matthews, J. B., Smith, J. A., and Hrnjez, B. J. (1997). *Am. J. Physiol.* **272**, C254–C262.
54. Matthews, J. B., Smith, J. A., Tally, K. J., Awtrey, C. S., Nguyen, H., Rich, J., and Madara, J. L. (1994). *J. Biol. Chem.* **269**, 15703–15709.
55. Merino, A., Kaplan, M. R., Hole, A. E., Hebert, S. C., and Gamba, G. (1995). *J. Am. Soc. Nephrol.* **6**, 346 (abstract).
56. Moore, M. L., George, J. N., and Turner, R. J. (1995). *Biochem. J.* **309**, 637–642.
57. Mount, D. B., Hall, A. E., Plata, C., Villanueva, Y., Kaplan, M. R., Gamba, G., and Hebert, S. C. (1995). *J. Am. Soc. Nephrol.* **6**, 347 (abstract).
58. Obermuller, N., Kunchaparty, S., Ellison, D. H., and Bachmann, S. (1996). *J. Clin. Invest.* **98**, 635–640.
59. O'Donnell, M. E. (1989). *J. Biol. Chem.* **264**, 20326–20330.
60. O'Donnell, M. E., Martinez, A., and Sun, D. (1995). *Am. J. Physiol.* **269**, C1513–C1523.
61. O'Neill, W. C., and Klein, J. D. (1992). *Am. J. Physiol.* **262**, C436–C444.
62. Palfrey, H. C., and O'Donnell, M. E. (1992). *Cell. Physiol. Biochem.* **2**, 293–307.
63. Payne, J. A., and Forbush, B., III (1994). *Proc. Natl. Acad. Sci. USA* **91**, 4544–4548.
64. Payne, J. A., Stevenson, T. J., and Donaldson, L. F. (1996). *J. Biol. Chem.* **271**, 16245–16252.
65. Payne, J. A., Xu, J.-C., Haas, M., Lytle, C. Y., Ward, D., and Forbush, B., III (1995). *J. Biol. Chem.* **270**, 17977–17985.
66. Pewitt, E. B., Hegde, R. S., Haas, M., and Palfrey, H. C. (1990). *J. Biol. Chem.* **265**, 20747–20756.
67. Plotkin, M. D., Kaplan, M. R., Peterson, L. N., Gullans, S. R., Hebert, S. C., and Delpire, E. (1997). *Am. J. Physiol.* **272**, C173–C183.
68. Poch, E., Suastegui, R., Gamba, G., and Hebert, S. C. (1996). *J. Am. Soc. Nephrol.* **7**, 1288 (abstract).
69. Prat, A. G., Bertorello, A. M., Ausiello, D. A., and Cantiello, H. F. (1993). *Am. J. Physiol.* **265**, C224–C233.
70. Quaggin, S. E., Payne, J. A., Forbush, B., III, and Igarashi, P. (1995). *Mammalian Genome* **6**, 557–558.
71. Robertson, M. A., and Foskett, J. K. (1994). *Am. J. Physiol.* **267**, C146–C156.
72. Russell, J. M. (1983). *J. Gen. Physiol.* **81**, 909–925.
73. Sachs, J. R., and Martin, D. W. (1993). *J. Gen. Physiol.* **102**, 551–573.
74. Shapiro, M., Matthews, J., Hecht, G., Delp, C., and Madara, J. L. (1991). *J. Clin. Invest.* **87**, 1903–1909.
75. Shorofsky, S. R., Field, M., and Fozzard, H. A. (1984). *J. Membr. Biol.* **81**, 1–8.
76. Simon, D. B., Karet, F. E., Hamdan, J. M., DiPietro, A., Sanjad, S. A., and Lifton, R. P. (1996). *Nature Genet.* **13**, 183–188.
77. Simon, D. B., and Lifton, R. P. (1996). *Am. J. Physiol.* **271**, F961–F966.
78. Simon, D. B., Nelson-Williams, C., Bia, M. J., Ellison, D., Karet, F. E., Molina, A. M., Vaara, I., Iwata, F., Cushner, H. M., Koolen, M., Gainza, F. J., Gitelman, H. J., and Lifton, R. P. (1996). *Nature Genet.* **12**, 24–30.
79. Sun, A., Grossman, E. B., Lombardi, M., and Hebert, S. C. (1991). *J. Membr. Biol.* **120**, 83–94.
80. Sun, A. M., Saltzberg, S. N., Kikeri, D., and Hebert, S. C. (1990). *Kidney Int.* **38**, 1019–1029.
81. Tanimura, A., Kurihara, K., Reshkin, S. J., and Turner, R. J. (1995). *J. Biol. Chem.* **270**, 25252–25258.
82. Torchia, J., Lytle, C., Pon, D. J., Forbush, B., III, and Sen, A. K. (1992). *J. Biol. Chem.* **267**, 25444–25450.
83. Treharne, K. J., Marshall, L. J., and Mehta, A. (1994). *Am. J. Physiol.* **267**, L592–L601.
84. Wiley, J. S., and Cooper, R. A. (1974). *J. Clin. Invest.* **53**, 745–755.
85. Wu, M. S., Bens, M., Cluzeaud, F., and Vandewalle, A. (1994). *J. Membr. Biol.* **142**, 323–336.
86. Xu, J.-C., Lytle, C., Zhu, T. T., Payne, J. A., Benz, E., Jr., and Forbush, B., III (1994). *Proc. Natl. Acad. Sci. USA* **91**, 2201–2205.
87. Yang, T., Huang, Y. G., Singh, I., Schnermann, J., and Briggs, J. P. (1996). *Am. J. Physiol.* **271**, F931–F939.
88. Yerby, T. R., Vibat, C. R. T., Sun, D., Payne, J. A., and O'Donnell, M. E. (1997). *Am. J. Physiol.* **273**, C188–C197.