J. Membrane Biol. 158, 177–186 (1997)

The Journal of
Membrane
Biology

Topical Review

The Molecular Physiology of Electroneutral Cation-Chloride Cotransport

D.B. Mount, R.S. Hoover, S.C. Hebert
Division of Nephrology, Department of Medicine, Vanderbilt University Medical Center, S-3223 Medical Center North, Nashville, TN 37232-2372, USA

Received: 14 February 1997

Abstract. The application of molecular biology to the study of electroneutral cation-chloride cotransporters has been extremely successful, resulting in the identification of a new gene family of five membrane proteins. The function, expression, and regulation of these important proteins can increasingly be described in molecular terms. In addition, mutations in two renal cation-chloride transporter genes have been found in patients with Bartter's and Gitelman's syndromes, autosomal recessive disorders of renal salt excretion.

Introduction

Electroneutral transport of sodium and/or potassium with chloride is a feature of multiple cell types, performing a variety of physiological roles [31, 36, 45, 50]. Despite differing in the identity and stoichiometry of the ions transported, these transporters share two main features: a dependence on the simultaneous presence of all the transported ions and differential sensitivity to thiazide or loop diuretics. Progress in this field has accelerated considerably since the molecular identification of the first members of the electroneutral cation-chloride cotransporter family, which now contains a total of five structurally homologous proteins (Table). The availability of specific antibodies and molecular probes has refined the understanding of these transporters, advances which are the primary subject of this review.

Correspondence to: S.C. Hebert

Cloning and Structure of the Cation-Chloride Transporters

It is perhaps fitting that the first chloride transporters were cloned from flounder urinary bladder and shark rectal gland, tissues which have long served as model epithelia. The flounder urinary bladder, essentially an extension of the kidney, absorbs Na-Cl through a K-independent electroneutral Na-Cl cotransporter which is sensitive to thiazides and related diuretics. A 3.7 kb cDNA encoding this cotransporter, denoted flTSC (flounder thiazide-sensitive cotransporter), was isolated by Gamba and coworkers by expression cloning in *Xenopus laevis* oocytes [19]. Oocytes injected with flTSC cRNA express a Cl-dependent uptake of ^{22}Na, with identical kinetics and pharmacology to that of the intact urinary bladder. A cDNA encoding the basolateral bumetanide-sensitive cotransporter from the shark rectal gland (NKCC1, for Na-K-Cl cotransporter) was also identified by expression cloning, using two monoclonal antibodies specific for the gland's 195 kDa bumetanide binding protein [95]. Heterologous expression of this cDNA in HEK 293 cells results in bumetanide-sensitive uptake of ^{86}Rb which is dependent on the presence of Na and Cl.

The cloning of mammalian cation-chloride cotransporters quickly followed. Using a 1.3 kb flTSC probe, two alternatively spliced cDNAs encoding the rat thiazide-sensitive cotransporter rTSC were cloned from rat renal cortex and expressed in *Xenopus* oocytes [20]. The same probe was also used to screen libraries from both rat and mouse outer medulla, identifying 4.6 kb cDNAs encoding the renal apical bumetanide-sensitive Na-K-2Cl cotransporter BSC1 (bumetanide-sensitive cotransporter) [20, 63]. Oocytes injected with cRNA from

Table 1. The five vertebrate cation chloride transporters

Cotransporter	Alternative nomenclature	Species	Size (amino acids	Chromosome	Genbank number
TSC	NCCT, SLC12A3	Flounder	1023		L11615
		Rat	1002		U10097
		Human	1021 (splice)	16q13	U44128
		Mouse	1002	8	U61085
BSC1	NKCC2, SLC12A1	Rat	1095		U10096
		Rabbit	1099		U07547
		Mouse	1095	2	U20975
			770 (splice)		U61381
		Human	1099	16	U58130
BSC2	NKCC1, SLC12A2	Shark	1191		U05958
		Mouse	1205	18	U13174
		Human	1212	5q23.3	U30246
KCC1		Human	1085	16q22.1	U55054
		Rat	1085		U55815
		Rabbit	1085		U55053
KCC2		Rat	1116		U55816

BSC1, BSC2 encode bumetanide-sensitive Na-K-2Cl cotransporters; TSC encodes a thiazide-sensitive Na-Cl cotransporter; KCC1, KCC2 encode K-Cl cotransporters.

both the mouse and rat BSC1 clones express bumetanide-sensitive, Na- and Cl-dependent uptake of ^{86}Rb. Homologous cDNAs, denoted NKCC2 by Forbush et al., have been cloned from rabbit [67] and human kidney [81]. In addition, cDNAs homologous to shark NKCC1, designated BSC2 in the alternative naming system, have been cloned from mouse [15] and human [68].

Several tissues and cells are known to express Na-independent and Cl-dependent transport of potassium by a transporter which is weakly sensitive to furosemide and bumetanide. By virtue of this physiology and pharmacology, K-Cl cotransporters were expected to be homologous to the diuretic-sensitive Na-(K)-Cl cotransporters [31], and indeed this assumption has turned out to be correct. Human ESTs (expressed sequence tags) moderately homologous to the known Na-(K)-Cl cotransporters were identified in the Genbank database and used to isolate cDNAs encoding the first K-Cl cotransporter, KCC1 [23]. Functional expression of full length rabbit KCC1 in HEK 293 cells confirmed that these cDNA clones encode K-Cl cotransporters (*see below*). A second putative K-Cl cotransporter, KCC2, was identified in rat brain by a similar cloning strategy [69].

In addition to the vertebrate clones mentioned above, more distantly related sequences have been identified in a moth, *C. elegans*, yeast, and a cyanobacterium [23, 45] (Fig. 1). All of the published full length sequences predict the same membrane topology, with 12 hydrophobic transmembrane (TM) domains flanked by cytoplasmic N- and C-terminal domains (Fig. 2). This membrane topology has yet to be confirmed in detail. However, the N-terminal domain of shark BSC2 is clearly phosphorylated [56], indicating an intracellular disposition, and preliminary evidence suggests that the C-terminal domain of rat BSC2 is phosphorylated by PKA [88]. In addition, mutation of the first of two predicted glycosylation sites in the extracellular loop between TM 7 and 8 of rat TSC dramatically reduces glycosylation of the protein, indicating an extracellular orientation [73]. Homology between family members is most marked in the TM domains, but is also evident in the C-terminal cytoplasmic domain and predicted intracellular loops. A major departure for the KCC proteins is the transposition of the large glycosylated extracellular loop to between TM 5 and 6 [Fig. 2]. Experimental evidence for glycosylation has been published for BSC2 [55, 57], TSC [19, 20], and KCC1 [23]. Although confirmation of the glycosylation of BSC1 has been difficult [20, 57], indirect evidence suggests that it is a glycoprotein [47].

Individual Cotransporters

BSC1

Function and Intrarenal Distribution

Expression of BSC1 is restricted to the kidney in all species which have been examined thus far. *In situ* hybridization detects transcript in the outer medulla and cortical medullary rays [20, 39, 65], indicating expression in the thick ascending limb of the loop of Henle

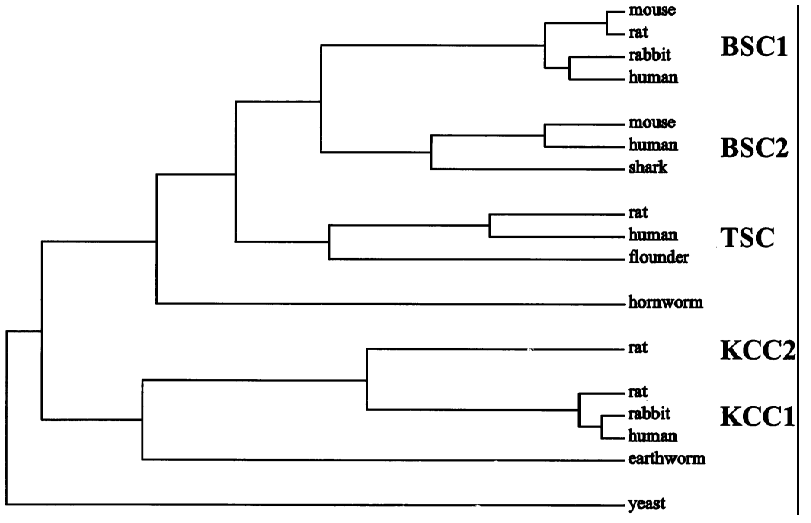

Fig. 1. The electroneutral cation-chloride cotransporter family. A homology tree of the cloned cotransporters based on amino acid sequences. The length of horizontal lines corresponds to evolutionary distance. Generated with the DNAStar program (Madison, WI).

(TALH). Affinity-purified antibodies to a C-terminal fusion protein of rBSC1 detect a protein of ~150 kDa in rat kidney [47], the same size as the major renal bumetanide binding protein [30]. This protein is detected at the apical cell membrane of TALH cells in both the outer medulla and cortex, and immunogold labeling with a separate BSC1-specific antibody indicates expression in a population of subapical vesicles [16]. In addition, BSC1 protein is present at the apical membrane of macula densa cells in both rat [47] and mouse kidney [46].

In the renal thick ascending limb, apical bumetanide-sensitive Na-K-2C1 transport plays a primary role in transcellular absorption of NaCl and plays an indirect role in paracellular transport of Na, Ca, Mg and other cations [28, 34]. Salt absorption by the medullary TALH is essential for countercurrent multiplication and the generation of medullary hypertonicity, a major prerequisite for excretion of a concentrated urine. BSC1 performs a similar function in the recycling of ammonium, since several studies indicate the NH_4 can substitute for K in transport by the apical Na-K-2Cl cotransporter of the TALH [26]. Ammonium generated by the proximal tubule from glutamine is thus reabsorbed by the TALH and concentrated in the renal medulla, from whence it is excreted in the urine.

The demonstration that BSC1 is expressed in the macula densa [46, 47, 65] is of particular interest, given the role of the macula densa plaque in both tubuloglomerular feedback (TGF) and tubular regulation of renin release. An increase in luminal NaCl at the macula densa decreases glomerular filtration via constriction of the afferent arteriole, a feedback mechanism which is blocked by luminal furosemide [42]. Increases in luminal NaCl inhibit renin release from juxtaglomerular cells in the afferent arteriole, an effect which is blocked by luminal bumetanide [53] and furosemide [33]. The macula densa primarily responds to changes in tubular

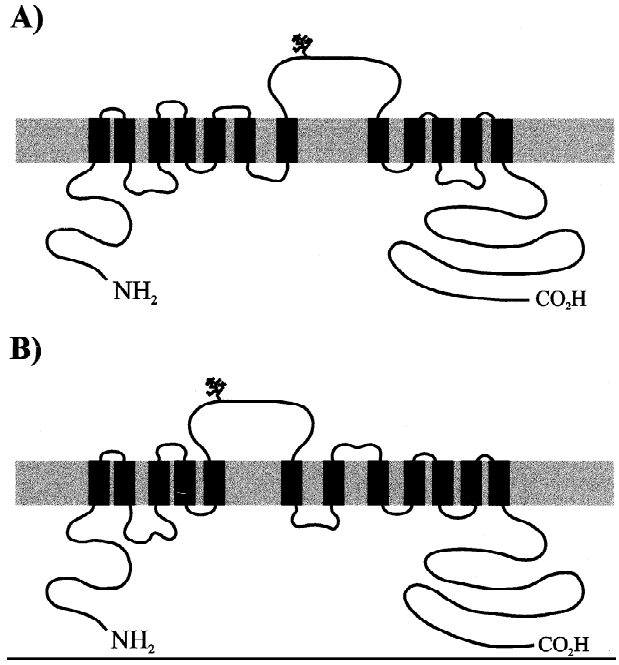

Fig. 2. Predicted membrane topology of the cation chloride cotransporters. All of the vertebrate clones predict a total of 12 hydrophobic membrane-spinning segments, flanked by large intracellular amino- and carboxy-terminal domains. The Na-(K)-Cl cotransporters (Fig. 2*A;* BSC1, BSC2 and TSC) and the K-Cl cotransporters (Fig. 2*B;* KCC1 and KCC2) differ in the predicted position of the large extracellular loop.

Cl, since variation in tubular Na alone has no effect on either TGF or renin secretion [53]. Macula densa cells have been shown to express apical bumetanide-sensitive Na-K-2Cl activity with an estimated K_m for Cl of 32.5 mM [50], close to the half-maximal concentration of Cl for modulation of renin release [33]. It is unclear how the initiating stimuli for TGF and renin release are trans-

mitted to the afferent arteriole and juxtaglomerular cells. However, both processes appear to be modulated by nitric oxide (NO) generated by the neuronal isoform of NO synthase [5, 43], an enzyme which is heavily expressed in the macula densa of several species [2].

Regulation of BSC1

The function and expression of BSC1 is likely regulated at multiple levels. The promoter of the BSC1 gene contains consensus sites for cAMP-response element binding proteins [40], hinting that cAMP may regulate transcription of BSC1. Alternative splicing of the rabbit, mouse and human BSC1 genes has been demonstrated, involving three mutually exclusive cassette exons [A, B, and F] near the 5′ end of the gene [39, 63, 67, 81]. Independent alternative splicing of the 3′ end of the mouse BSC1 transcript has also been described [63], resulting in a different 3′ UTR and truncation of the C-terminal cytoplasmic domain (*see below*). The cassette exons are predicted to encode 31 amino acids, spanning most of the second transmembrane domain and extending 10–13 amino acids into the cytoplasm. This cytoplasmic extension is strongly conserved between the three cassettes. Expression of the A, B, and F cassettes is spatially restricted along the TALH (in outer stripe, cortex, and inner stripe, respectively) [39, 67]. This pattern suggests that alternative splicing of the cassette exons generates axial heterogeneity in ion transport. However, expression problems have prevented functional confirmation of this hypothesis.

Multiple hormones regulate NaCl transport in the TALH, through a combination of both primary and secondary effects on apical Na-K-2Cl cotransport (reviewed in references 28, 34, 45). Vasopressin appears to directly activate apical Na-K-2Cl cotransport [61], although investigators have also argued for secondary effects of basolateral Cl channel activation [28]. In the mouse medullary TALH, vasopressin induces a switch in the K-dependence of the apical Na-K-2Cl transporter, from a completely K-independent Na-Cl mode to a K-dependent Na-K-2Cl mode [86]. Recycling of K through the Na-K-2Cl cotransporter and apical K channels generates a lumen-positive potential difference, which drives paracellular absorption of cations through the cation-selective paracellular pathway [34]. This arrangement is largely responsible for the absorption of divalent cations by the TALH, which reclaims ~20% of filtered Ca and ~50% of filtered Mg [28]. It is also more efficient than pure transcellular transport, increasing transepithelial Na absorption by 50% without an increase in energy expenditure [86]. K-independent bumetanide-sensitive Na-Cl cotransport is not restricted to mouse TALH, having also been described in both rabbit [1] and rat kidney [54].

All of the BSC1 cDNAs predict consensus phosphorylation sites for both PKA and PKC. However, unlike BSC2 (*see below*), there is no published evidence that BSC1 is a phosphoprotein. Alternative splicing of the 3′ end of mBSC1 results in a truncated C-terminus, with a unique 55-amino acid C-terminal sequence which differs in the inclusion of phosphorylation sites for both PKA and PKC [63]. Differential inclusion of phosphorylation sites due to alternative splicing has not been described in other Na-dependent cotransporters, but is known to occur in ion channels [89]. It is as yet unclear whether this shorter mBSC1 isoform encodes the K-independent Na-Cl cotransporter [86] and/or the ~75 kDa low affinity bumetanide binding protein of mouse outer medulla [30].

BSC2

Expression and Function of BSC2

Since the initial description of bumetanide-sensitive Na-K-2Cl cotransport in Ehrlich cells [21], this activity has been detected in a wide variety of cultured cells [31]. However, the relevance of some of these latter findings to native tissue has been recently questioned. BSC2 transcript and Na-K-2Cl cotransport are not present in freshly isolated proximal tubule cells, vascular smooth muscle cells, and aortic endothelial cells, but are strongly induced after more prolonged culture [74]. Independent observations suggest that this induction of BSC2 is involved in the increase in cell volume during cell growth and progression through the cell cycle, analogous to the well-known stimulation of Na-K-2Cl cotransport by hypertonic conditions [36]. Thus the K content and cell volume of NIH 3T3 cells increase in parallel after serum stimulation, and bumetanide decreases both measured cell volume and cell number in response to mitogens [10]. Influx of Na through both amiloride- and bumetanide-sensitive pathways is also an early event after serum stimulation of 3T3 cells, followed by efflux through Na-K-ATPase [6].

The disparity between cell culture and tissue notwithstanding, BSC2 is widely expressed [15, 68]. The precise localization within tissues has been thoroughly studied in kidney and brain [46, 72]. These studies utilized a BSC2-specific antibody generated against a 74-amino acid C-terminal fusion protein. This antibody recognizes two proteins of ~145 kDa and ~155 kDa, presumably differentially glycosylated forms of BSC2, in both mouse kidney and rat brain. Within the kidney, basolateral staining of BSC2 is detected in the terminal two thirds of the inner medullary collecting duct (IMCD), as predicted by the functional evidence for basolateral Na-K-2Cl transport in freshly isolated rat papilla cells [29] and in a mouse IMCD cell line (mIMCD-3) [15]. BSC2 expression in the IMCD may regulate cell volume, given the wide fluctuations of extracellular os-

molality in this nephron segment. It also appears to play a role in ANP-induced NaCl secretion [78]. In addition, BSC2 in the IMCD probably provides a basolateral entry pathway for the NH_4 ion, which is transported by the basolateral Na-K-2Cl cotransporter in the mIMCD-3 cell line [94]. Thus both BSC1 and BSC2 are implicated in countercurrent multiplication and excretion of ammonium.

Within the glomerulus BSC2 expression is detected in mesangial cells [46], which express a Na-K-2Cl cotransporter activity in culture which is modulated by vasopressin, angiotensin II, and ANP [37]. Unexpected staining of juxtaglomerular smooth muscle cells in the afferent arteriole is also evident, however no other vascular cells are stained in the kidney. Extraglomerular cells adjacent to the macula densa, likely extraglomerular mesangium, are also strongly positive [46]. The function of BSC2 in these structures is completely unknown. However, an attractive hypothesis is that BSC2 participates in propagation of the chloride signal ''sensed'' in the macula densa. In this regard, a reduction in extracellular Cl stimulates a rise in intracellular Ca and release of NO in rat mesangial cells [90].

Within the rat brain BSC2 expression is particularly prominent in the choroid plexus, where it colocalizes with Na-K-ATPase at the apical CSF (cerebrospinal fluid) cell membrane [72]. Apical bumetanide-sensitive ^{86}Rb uptake is also detectable in primary cultures of choroid plexus. This apical localization agrees with some but not all models of choroid plexus ion transport [44]. An *in vivo* role for Na-K-2Cl cotransport has been difficult to ascertain, however intracisternal perfusion of bumetanide appears to inhibit CSF production by as much as 50% [44].

BSC2 protein is also detected in neuronal cell bodies, and staining is particularly intense within dorsal root sensory neurons in the peripheral nervous system. Immunoreactivity is notably absent in glial cells and brain capillary endothelial cells, in which Na-K-2Cl cotransport has been well documented in culture. Within neurons BSC2 likely helps generate and maintain the transmembrane Cl concentration gradient. Both outward and inward Cl transport have been implicated in the differential response of neurons to $GABA_A$ receptor activation and other stimuli [60]. In cells with strong inward transport of Cl (likely through BSC2), intracellular Cl concentration is high and $GABA_A$ receptor stimulation, which activates a Cl conductance, is depolarizing and excitatory. In contrast, in neurons with outward Cl transport (possibly K-Cl cotransport), $GABA_A$ activation is hyperpolarizing and inhibitory. Intracellular chloride activity thus plays a central role in neuronal physiology [84].

With respect to secretory epithelia, BSC2 immunoreactivity has been localized to the basolateral membrane of rat parotid gland. This study used broadly specific anti-BSC monoclonal antibodies generated against a large C-terminal fusion protein of human BSC2 [56]. Large amounts of BSC2 transcript have been detected in stomach [15], prompting an examination of its role in gastric acid secretion [83]. Expression of BSC2 is particularly prominent in the *Necturus* gastric fundus, essentially doubling after feeding. The effects of basolateral bumetanide and ion substitution strongly support a functional role for BSC2 in the secretion of stomach acid, likely by providing a basolateral entry pathway for Cl [83].

Regulation of BSC2

Bumetanide-sensitive Na-K-2Cl cotransport is affected by a wide variety of hormones, conditions, and second messengers. This issue has been recently reviewed [31, 45], and emphasis here is on particularly new developments. Evidence that BSC2 is regulated by phosphorylation/dephosphorylation is multiple, and BSC2 has been shown to be a phosphoprotein in several species and tissues [56, 72, 88]. The primary structure of human and mouse BSC2 contains one predicted PKA site and several PKC sites. A fraction of mBSC2 transcripts in brain are missing exon 21, a 48 bp exon which contains the putative PKA site; no other alternative splicing events were detected in a comprehensive survey of several tissues by RT-PCR [75]. One-dimensional phosphopeptide mapping of BSC2 protein from rat parotid indicates at least three separate phosphorylation sites, with differential labeling of one phosphopeptide after stimulation with isoproterenol [88]; the precise residues phosphorylated by specific kinases have not yet been defined.

The shark BSC2 protein sequence does not contain any putative PKA sites, yet in response to activation of PKA this protein is phosphorylated on a threonine which is not part of a consensus PKA site [56]. However, the stimulatory effect of PKA is blocked by raising internal Cl (Cl_i). This suggests that PKA does not directly phosphorylate shark BSC2, but rather activates a Cl-sensitive phosphorylation event, most likely by activating Cl extrusion through a PKA-sensitive conductive pathway such as CFTR [58]. Chloride may therefore regulate its own traffic through secretory epithelia by affecting phosphorylation of BSC2. This has been clearly demonstrated in both shark rectal gland and in dog tracheal epithelium, since in both systems depletion of Cl_i induces phosphorylation and activation of BSC2 [32, 58]. The identity of this Cl-sensitive kinase (or phosphatase) is unknown. Also unclear is the relative contribution of a proposed intracellular anion-modifier site on BSC2 [62], which could either directly modulate the cotransporter or regulate its accessibility for phosphorylation/dephosphorylation [58].

TSC

Expression Pattern and Function

In the mammalian kidney the distal convoluted tubule (DCT) reabsorbs 5–7% of the filtered sodium chloride load, almost exclusively through a thiazide-sensitive, K-independent Na-Cl cotransporter (TSC). In addition to its natriuretic effect, luminal thiazide in the early DCT is significantly hypocalciuric [13], suggesting that TSC is also involved in calcium homeostasis. Indeed, thiazides are a treatment option in the management of idiopathic hypercalciuria, a common cause of renal stones [66]. The role of TSC in DCT calcium handling has been addressed using an immortalized cell line from mouse DCT, in which apical thiazide increases intracellular calcium (Ca_i) [22]. The blockade of apical NaCl cotransport by thiazide decreases internal cell Cl, due to continued efflux of Cl through basolateral Cl channels. Cells treated with thiazide hyperpolarize toward the K equilibrium potential; this hyperpolarization increases Ca_i, which enters through dihydropyridine-sensitive apical Ca channels. The identity of the relevant basolateral Cl and apical Ca channels in the DCT is unknown.

Prior to the cloning of TSC it was demonstrated by *in vivo* microperfusion studies that the early distal tubule was the predominant site of thiazide-sensitive sodium chloride cotransport in rat kidney [13]. However, interspecies heterogeneity and the lack of distinct boundaries between the DCT and connecting tubule (CNT) made precise localization of this transport difficult. TSC-specific immunofluorescence [71] and *in situ* hybridization [3, 64] have been very useful in this regard. Affinity-purified antibodies to an amino-terminal fusion protein of rTSC detect three proteins of 135,140 and 155 kDa in rat renal cortex; this heterogeneity may reflect either differential glycosylation or alternative splicing. Immunofluorescence detects TSC protein at the apical cell membrane of the DCT, and confocal and electron microscopy also demonstrate TSC in subapical vesicles [71].

The phenotypic transition between cells of the CTAL (positive for BSC1 and Tamm-Horsfall glycoprotein) and the DCT (positive for TSC) is dramatic, occurring at a variable distance after the macula densa [64, 71]. In contrast, in the rat and human kidney the distal border of the DCT is much less abrupt, such that the CNT is composed of a mixture of TSC-positive cells, TSC-negative CNT cells and TSC-negative intercalated cells [64, 71]. The transition between the DCT and the CNT is more distinct in the rabbit [3]. Of relevance to the role of TSC in calcium homeostasis is the presence of calbindin D28, an intracellular calcium-binding protein implicated in transcellular calcium transport, in all cells expressing TSC [71]. In addition, within the late DCT and early CNT, some cells with apical TSC also express the basolateral Na/Ca exchanger and Ca-ATPase [64, 71].

Although flounder TSC is expressed in several tissues besides urinary bladder [19], northern blot analysis suggests that rTSC is kidney-specific [20]. A northern blot survey of human tissues using a human TSC probe is suggestive of extra-renal expression of hTSC, with a very weak 4.5 kb band in poly-$(A)^+$-RNA from small intestine, placenta, prostate, colon and spleen [11]. However, the molecular identity of this extra-renal transcript has not been established, and extra-renal expression of hTSC has not been confirmed by other investigators [59]. Extra-renal effects of thiazides are well-documented both physiologically and clinically, but are not clearly due to inhibition of TSC. For example, the effect of thiazide on Na-Cl cotransport in some epithelial tissues is due to inhibition of carbonic anhydrase and the resultant blockade of parallel apical Na-H and Cl-CO_3 exchange [25]. Thiazides are clinically associated with impaired glucose tolerance, and experimentally hydrochlorothiazide reduces insulin release from islet cells [70]. Preliminary evidence suggests that rat endocrine pancreas expresses rTSC, suggesting that TSC may play a direct role in modulating insulin release [7]. However, other explanations for the effect of thiazides on the pancreas are equally plausible [70]. Another well-described clinical effect of thiazides is an increase in bone density, and both thiazides and PTH increase Ca_i in rat UMR-106 cells, an osteoblast-like cell line which expresses rTSC transcript and protein [4]. The issue of *in vivo* expression of TSC in bone has not yet been addressed.

Regulation of TSC

Unlike Na-K-2Cl and K-Cl cotransport, there is little direct information on the regulation of thiazide-sensitive Na-Cl cotransport. Estrogen has been shown to regulate thiazide binding sites in rat kidney, such that females have twice the density of binding sites than males [12]. Estrogen affects renal sodium handling, and ovariectomized female rats have increased sodium excretion when compared to controls. Treatment with estrogen dramatically increases apical labeling of TSC and the content of immunoreactive TSC in the DCT of ovariectomized rats [92]. Whether this is due to a direct transcriptional effect has not been established. Moreover, the overall ultrastructure of DCT cells is affected by estrogen, with an increase in the number of apical microprojections [92].

Alternative splicing of TSC appears to be complex. The two rTSC cDNAs reported initially have identical open reading frames but different 3′ UTRs [20]. At least three other alternatively spliced isoforms of rTSC exist, differing dramatically in the 3′ end of the

open reading frame [38]. The functional effect of this alternative splicing is not yet clear.

Rat TSC contains five potential PKC phosphorylation sites and no consensus PKA sites. However, TSC has not yet been shown to be a phosphoprotein, and the effect in the DCT of hormones which activate PKC is not known. Receptors likely to regulate TSC via PKC include the extracellular Ca-sensing receptor, the PTH/PTHrP receptor, and the 5-HT_{1A} receptor, all of which are expressed in the DCT [76, 77].

In contrast to the paucity of information regarding the regulation of TSC, several studies have addressed the modulation of DCT structure and function by changes in luminal delivery of NaCl [34, 52, 85]. Increases in NaCl delivery to the DCT, induced by either increased dietary salt or furosemide treatment, are generally associated with cell hypertrophy, increased binding of thiazides, and increases in tubular transport capacity. Treatment of rats with furosemide also appears to increase the amount of rTSC transcript in the DCT [64]. Conversely, thiazide treatment and dietary NaCl restriction are associated with decreases in DCT transport capacity. Thiazide treatment of rats causes dose-dependent apoptosis of the DCT [52]. These morphological changes are accompanied by drastic decreases in the amount of TSC protein and transcript in the DCT. TSC-positive cells in the CNT, which likely have additional apical pathways for sodium entry, are spared the effect of thiazide; this distinction argues for a direct effect of impaired NaCl entry in DCT cells rather than a toxic effect of the drug. Remodelling of the DCT by hypertrophy or apoptosis may thus occur in response to changes in luminal NaCl delivery [85].

KCC1

Electroneutral cotransport of KCl is detected in a wide range of cells, functioning in both regulatory volume decrease [36] and in transepithelial salt transport. Consistent with this broad functional distribution, a 3.8 kb KCC1 transcript is heavily expressed in multiple tissues, including brain. A second less abundant transcript of ~4.4 kb is also detected in a subset of tissues [23]. Once KCC1-specific antibodies are developed the expression pattern within tissues can begin to be addressed. Of particular interest is the role of KCC1 in basolateral [34] and apical [91] KC1 flux in the kidney.

Stable expression of full length rabbit KCC1 in HEK 293 cells [23] indicates the expected characteristics of a K-Cl cotransporter [50]. Thus ^{86}Rb efflux in these cells is stimulated by cell swelling, to a much greater extent than in untransfected cells. Chloride-dependent influx of ^{86}Rb is activated by treatment with NEM, a reagent which likely exerts its effect by modification of sulfhydryl groups on the cotransporter and/or associated proteins. This influx is inhibited by furosemide and bumetanide, with K_i values of 40 μM and 59 μM, respectively, much higher than the equivalent values for human BSC2 [23].

The primary structure of all three mammalian KCC1 proteins contain consensus phosphorylation sites for PKC, suggesting regulation by phosphorylation/dephosphorylation. Regulation of KCC1 is likely complex, however red cell K-C1 cotransport is known to be stimulated by kinase inhibition and inactivated by phosphatase inhibition [17, 50]. The response of K-Cl transport is thus opposite to that of BSC2, as predicted by their roles in regulatory volume decrease and increase, respectively.

KCC2

In contrast to KCC1, KCC2 is expressed only in the brain, at least by northern blot analysis [69]. *In situ* hybridization reveals expression in neurons throughout the central nervous system, suggesting a role in the regulation of neuronal Cl_i (*see* discussion of BSC2 function in the brain). Functional expression of KCC2 has not been reported, and nothing is known about its regulation. However, the primary sequence of KCC2 predicts a solitary tyrosine kinase phosphorylation site and several PKC sites.

Relevance of Cation-Chloride Transporters to Human Disease

The human BSC1 and TSC genes have been linked to two hereditary forms of hypokalemic alkalosis, Bartter's syndrome and Gitelman's syndrome, providing a dramatic demonstration of their physiological importance. Patients with classic Bartter's syndrome have a decreased concentrating ability and polyuria. In addition to hypokalemia and metabolic alkalosis, they frequently exhibit increased urinary excretion of calcium with a normal serum magnesium level [8]. This constellation of findings is most compatible with a primary defect in the TALH, and the concordance between Bartter's syndrome and the pathophysiological consequences of loop diuretic therapy implicated BSC1 as the gene. Homozygous disease-causing mutations in the human BSC1 gene have recently been reported in several kindreds with classic Bartter's syndrome [81], however the absence of BSC1 mutations in other families suggested genetic heterogeneity. The subsequent demonstration of disease-associated mutations in the human ROMK K channel gene [41, 82], which encodes a component of the apical K conductance of the TALH, genetically demonstrates

the functional coupling between apical K channels and BSC1.

Gitelman's syndrome is a more common and benign cause of hypokalemia than classic Bartter's syndrome [24]. A major distinguishing feature of Gitelman's syndrome is the presence of marked hypomagnesemia and hypocalcuria [8], without impairment in urinary concentrating ability. The occurrence of hypocalcuria, along with the absence of a diuretic response to thiazides, pointed to a defect in TSC; this has been confirmed by the direct characterization of nonconservative mutations in affected patients [80]. Unlike Bartter's syndrome, Gitelman's appears to be genetically homogeneous. An intriguing observation is the apparently high frequency of compound heterozygotes, suggesting that mutant hTSC alleles may be relatively common in the general population.

Gordon's syndrome, described as the ''mirror image'' of Bartter's syndrome [27], consists of autosomal dominant hypertension and hyperkalemia, with suppression of the renin-angiotensin-aldosterone axis and a hyperchloremic acidosis. The full phenotype can be reversed by aggressive salt restriction [48]. Further characterization has demonstrated hyperabsorption of NaCl, but not Na accompanied by other anions. This observation and the beneficial therapeutic effect of thiazides suggested overactivity of TSC [87]. This was a particularly attractive hypothesis, given the precedence of both inactivating and activating mutations in renal tubular disorders, in the human Ca-sensing receptor [9] and in subunits of ENaC, the amiloride-sensitive epithelial Na channel [51]. However, TSC has been excluded as the locus for Gordon's syndrome in several affected families [79 and M. Pollak, *personal communication*].

The involvement of TSC, BSC1 and associated proteins in genetic disorders of renal salt handling underscores the primacy of the kidney in the regulation of blood pressure and extracellular volume [51]. Subtle variation in these genes or in the regulatory mechanisms governing the cotransporters may underlie ''essential'' hypertension and/or affect the response to therapy. In addition, modulation of BSC1 and TSC may contribute to disorders of calcium homeostasis such as nephrolithiasis and osteoporosis. The other cation-chloride transporters are also likely to play a role in human pathophysiology. For example, excessive K-Cl cotransport may play a role in the dehydration of young red cells in sickle cell anemia [18]. Increases in erythrocyte magnesium in a transgenic model of sickle cell disease have been shown to decrease K-Cl cotransport and increase hemoglobin levels [14]. The contribution of KCC1, KCC2, and BSC2 to neurological disorders such as epilepsy merits particular investigation [35]. Finally, bumetanide-sensitive Na-K-2Cl cotransport mediates the increase in cell volume induced by HIV infection of T-cell cell lines, and both furosemide and bumetanide block HIV production and the cytopathic effects of HIV in culture [93].

Conclusion

In the four years since the first reported cloning of an electroneutral cation-chloride transporter progress has been most gratifying. The recent addition of the K-Cl cotransporters to the gene family fulfills predictions which followed the initial cloning efforts [31]. Localization studies have been informative, particularly for BSC2, which was expected to have a broader expression pattern. The literature regarding bumetanide-sensitive Na-K-2Cl cotransport in cultured cells will need to be re-appraised in light of the observation that cell culture induces BSC2 in cells which do not normally express it *in vivo* [46, 72, 74]. Molecular techniques can now be directed at specific inhibition and germline inactivation of the cotransporters, resulting in a more precise understanding of the individual family members. This approach will hopefully clarify the relative roles of chloride channels and cation-chloride cotransporters in the maintenance of neuronal transmembrane chloride gradients [84]. The molecular description of phosphorylation sites, associated proteins, ion- and drug-binding sites is hopefully pending. This understanding will ultimately result in the development of more specific inhibitors of the individual transporters, agents which are likely to have broad clinical and experimental utility.

Original research was supported by grants from the National Institutes of Health to SCH (DK45792 and DK36803) and to DBM (DK02328). We are grateful to Eric Delpire for pre-prints and helpful comments.

References

1. Alvo, M., Calamia, J., Eveloff, J. 1985. *Am. J. Physiol.* **249:**F34–39
2. Bachmann, S., Bosse, H.M., Mundel, P. 1995. *Am. J. Physiol.* **268:**F885–898
3. Bachmann, S., Velazquez, H., Obermüller, N., Reilly, R.F., Moser, D., Ellison, D.H. 1995. *J. Clin. Invest.* **96:**2510–2514
4. Barry, E.L.R., Gesek, F.A., Kaplan, M.R., Hebert, S.C., Friedman, P.A. 1997. *Am. J. Physiol.* (*in press*)
5. Beierwaltes, W.H. 1995. *Am. J. Physiol.* **269:**F134–139
6. Berman, E., Sharon, I., Atlan, H. 1995. *Biochim. Biophys. Acta* **1239:**177–1785
7. Bernstein, P.I., Zawalach, W., Bartiss, A., Reilly, R., Palcso, M., Ellison, D.H. 1995. *J. Am. Soc. Nephrol.* **6:**732
8. Bettinelli, A., Bianchetti, M.G., Girardin, E., Caringella, A., Cecconi, M., Appiani, A.C., Pavanello, L., Gastaldi, R., Isimbaldi, C., Lama, G., 1992. *J. Pediatr.* **120:**38–43
9. Brown, E.M., Pollak, M., Seidman, C.E., Seidman, J.G., Chou, Y.H., Riccardi, D., Hebert, S.C. 1995. *N. Engl. J. Med.* **333:**234–240

10. Bussolati, O., Uggeri, J., Belletti, S., Dall'Asta, V., Gazzola, G.C. 1996. *FASEB J.* **10:**920–926
11. Chang, H., Tashiro, K., Hirai, M., Ikeda, K., Kurokawa, K., Fujita, T. 1996. *Biochem. Biophys. Res. Comm.* **223:**324–328
12. Chen, Z., Vaughn, D.A., Fanestil, D.D. 1994. *J. Am. Soc. Nephrol.* **5:**1112–1119
13. Costanzo, L.S. 1985. *Am. J. Physiol.* **248:**F527–535
14. De Franceschi, L., Beuzard, Y., Jouault, H., Brugnara, C. 1996. *Blood* **88:**2738–2744
15. Delpire, E., Rauchman, M.I., Beier, D.R., Hebert, S.C., Gullans, S.R. 1994. *J. Biol. Chem.* **269:**25677–25683
16. Ecelbarger, C.A., Wade, J.B., Terris, J., Marples, D., Nielsen, S., Knepper, M.A. 1995. *J. Am. Soc. Nephrol.* **6:**335
17. Flatman, P.W., Adragna, N.C., Lauf, P.K. 1996. *Am. J. Physiol.* **271:**C255–C263
18. Franco, R.S., Palascak, M., Thompson, H., Joiner, C.H. 1995. *J. Clin. Invest.* **95:**2573–2580
19. Gamba, G., Saltzberg, S.N., Lombardi, M., Miyanoshita, A., Lytton, J., Hediger, M.A., Brenner, B.M., Hebert, S.C. 1993. *Proc. Natl. Acad. Sci. USA* **90:**2749–2753
20. Gamba, G., Miyanoshita, A., Lombardi, M., Lytton, J., Lee, W.S., Hediger, M.A., Hebert, S.C. 1994. *J. Biol. Chem.* **269:**17713–17722
21. Geck, P., Pietrzyk, C., Burckhardt, B.C., Pfeiffer, B., Heinz, E. 1980. *Biochim. Biophys. Acta* **600:**432–447
22. Gesek, F.A., Friedman, P.A. 1992. *J. Clin. Invest.* **90:**429–438
23. Gillen, C.M., Brill, S., Payne, J.A., Forbush, B., 3rd. 1996. *J. Biol. Chem.* **271:**16237–16244
24. Gladziwa, U., Schwarz, R., Gitter, A.H., Bijman, J., Seyberth, H., Beck, F., Ritz, E., Gross, P. 1995. *Nephrol. Dial. Transplant* **10:**1607–1613
25. Goldfarb, D.S., Chan, A.J., Hernandez, D., Charney, A.N. 1991. *Am. J. Physiol.* **261:**F452–F458
26. Good, D.W. 1994. *Anm. Rev. of Physiol.* **56:**623–647
27. Gordon, R.D. 1986. *Hypertension* **8:**93–102
28. Greger, R. 1985. *Physiol. Rev.* **65:**760–797
29. Grupp, C., Pavenstadt-Grupp, I., Grunewald, R.W., Bevan, C., Stokes, J.B., Kinne, R.K. 1989. *Kidney Int.* **36:**201–209
30. Haas, M., Dunham, P.B., Forbush, B. 3rd. 1991. *Am. J. Physiol.* **260:**C791–C804
31. Haas, M. 1994. *Am. J. Physiol.* **267:**C869–C885
32. Haas, M., McBrayer, D., Lytle, C. 1995. *J. Biol. Chem.* **270:**28955–28961
33. He, X.-R., Greenberg, S.G., Briggs, J.P., Schnermann, J. 1995. *Hypertension* **26:**137–142
34. Hebert, S.C. 1992. *In:* Handbook of Physiology—Renal Physiology. pp. 875–925. Oxford University Press
35. Hochman, D.W., Baraban, S.C., Owens, J.W., Schwartzkroin, P.A. 1995. *Science* **270:**99–102
36. Hoffmann, E.K., Dunham, P.B. 1995. *Int. Rev. Cytol.* **161:**173–262
37. Homma, T., Hoover, R.L., Harris, R.C. 1990. *Am. J. Physiol.* **258:** C862–C870
38. Hoover, R.S., Mount, D.B., Gamba, G., Hebert, S.C. 1996. *J. Am. Soc. Nephrol.* **7:**1281
39. Igarashi P., Vanden Heuvel, G.B., Payne, J.A., Forbush, B., 3rd. 1995. *Am. J. Physiol.* **269:**F405–F418
40. Igarashi, P., Whyte, D.A., Li, K., Nagami, G.T. 1996. *J. Biol. Chem.* **271:**9666–9674
41. International Collaborative Group. 1997. *Hum. Mol. Genet.* **6:**17–26
42. Ito, S., Carretero, O.A. 1990. *Kidney Int.* **38:**1206–1210
43. Ito, S., Carretero, O.A., Abe, K. 1996. *Kidney Int.* 49, Suppl. **55:**S6–S8
44. Javaheri, S., Wagner, K.R. 1993. *J. Clin. Invest.* **92:**2257–2261
45. Kaplan, M.R., Mount, D.B., Delpire, E., Gamba, G., Hebert, S.C. 1996. *Ann. Rev. Physiol.* **58:**649–668
46. Kaplan, M.R., Plotkin, M.D., Brown, D., Hebert, S.C., Delpire, E. 1996. *J. Clin. Invest.* **98:**723–730
47. Kaplan, M.R., Plotkin, M.D., Lee, W.S., Xu, Z.C., Lytton, J., Hebert, S.C. 1996. *Kidney Int.* **49:**40–47
48. Klemm, S.A., Gordon, R.D., Tunny, T.J., Finn, W.L. 1990. *Clin. Exp. Pharmacol. Physiol.* **17:**191–205
49. Lapointe, J.Y., Laamarti, A., Hurst, A.M., Fowler, B.C., Bell, P.D. 1995. *Kidney Int.* **47:**752–757
50. Lauf, P.K., Bauer, J., Adragna, N.C., Fujise, H., Zade-Oppen, A.M., Ryu, K.H., Delpire, E. 1992. *Am. J. Physiol.* **263:**C917–C932
51. Lifton, R.P. 1996. *Science* **272:**676–680
52. Loffing, J., Loffing-Cueni, D., Hegyi, I., Kaplan, M.R., Hebert, S.C., Le Hir, M., Kaissling, B. 1996. *Kidney Int.* **50:**1180–1190
53. Lorenz, J.N., Weihprecht, H., Schnermann, J., Skott, O., Briggs, J.P. 1991. *Am. J. Physiol.* **260:**F486–F493
54. Ludens, J.H., Clark, M.A., Lawson, J.A. 1995. *J. Am. Soc. Nephrol.* **6:**344
55. Lytle, C., Xu, J.C., Biemesderfer, D., Haas, M., Forbush, B.3rd. 1992. *J. Biol. Chem.* **267:**25428–25437
56. Lytle, C., Forbush, B.3rd. 1992. *J. Biol. Chem.* **267:**25438–25443
57. Lytle, C., Xu, J.C., Biemesderfer, D., Forbush, B.3rd. 1995. *Am. J. Physiol.* **269:**C1496–C1505
58. Lytle, C., Forbush, B.3rd. 1996. *Am. J. Physiol.* **270:**C437–C448
59. Mastroianni, N., De Fusco, M., Zollo, M., Arrigo, G., Zuffardi, O., Bettinelli, A., Ballabio, A., Casari, G. 1996. *Genomics* **35:**486–493
60. Misgeld, U., Deisz, R.A., Dodt, H.U., Lux, H.D. 1986. *Science* **232:**1413–1415
61. Molony, D.A., Reeves, W.B., Hebert, S.C., Andreoli, T.E. 1987. *Am. J. Physiol.* **252:**F177–187
62. Moore, M.L., George, J.N., Turner, R.J. 1995. *Biochem. J.* **309:**637–642
63. Mount, D.B., Hall, A.E., Plata, C., Vilaneuva, Y., Kaplan, M.R., Gamba, G., Hebert, S.C. 1995. *J. Am. Soc. Nephrol.* **6:**347
64. Obermüller N., Bernstein, P., Velazquez, H., Reilly, R., Moser, D., Ellison, D.H., Bachmann, S. 1995. *Am. J. Physiol.* **269:**F900–F1010
65. Obermüller, N., Kunchaparty, S., Ellison, D.H., Bachmann, S. 1996. *J. Clin. Invest.* **98:**635–640
66. Parks, J.H., Coe, F.L. 1996. *Seminars in Nephrology* **16:**398–411
67. Payne, J.A., Forbush, B.3rd. 1994. *Proc. Natl. Acad. Sci. USA* **91:**4544–4548
68. Payne, J.A., Xu, J.C., Haas, M., Lytle, C.Y., Ward, D., Forbush, B.3rd. 1995. *J. Biol. Chem.* **270:**17977–17985
69. Payne, J.A., Stevenson, T.J., Donaldson, L.F. 1996. *J. Biol. Chem.* **271:**16245–16252
70. Pickkers, P., Schachter, M., Sever, P.S., Hughes, A.D. 1995. *J. Hum. Hypertens.* **9:**691–693
71. Plotkin, M.D., Kaplan, M.R., Verlander, J.W., Lee, W.-S., Brown, D., Poch, E., Gullans, S.R., Hebert, S.C. 1996. *Kidney Int.*. **50:**174–183
72. Plotkin, M.D., Kaplan, M.R., Peterson, L.N., Gullans, S.R., Hebert, S.C., Delpire, E. 1997. *Am. J. Physiol.* **272:**C173–C183
73. Poch, E., Suastegui, R., Gamba, G., Hebert, S.C. 1996. *J. Am. Soc. Nephrol.* **7:**1288
74. Raat, N.J., Delpire, E., van Os, C.H., Bindels, R.J. 1996. *Pfleugers Arch.* **431:**458–460
75. Randall, J., Thorne, T., Delpire, E. 1996. *FASEB J.* **10:***A*89 (*Abstr.*)
76. Raymond, J.R., Kim, J., Beach, R.E., Tisher, C.C. 1987. *Am. J. Physiol.* **264:**F9–F19
77. Riccardi, D., Lee, W.-S., Lee, K., Segre, G.V., Hebert, S.C. 1996. *Am. J. Physiol.* **271:**F951–F956

78. Rocha, A.S., Kudo, L.H. 1990. *Am. J. Physiol.* **259:**F258–268
79. Simon, D.B., Farfel, Z., Ellison, D., Bia, M., Tucci, J., Lifton, R.P. 1995. *J. Am. Soc. Nephrol.* **6:**632
80. 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., Gitleman, H.J., Lifton, R.P. 1996. *Nat. Genet.* **12:**24–30
81. Simon, D.B., Karet, F.E., Hamdan, J.M., DiPietro, A., Sanjad, S.A., Lifton, R.P. 1996. *Nat. Genet.* **13:**183–188
82. Simon, D.B., Karet, F.E., Rodriquez-Soriano, J., Hamdan, J.H., DiPietro, A., Trachtman, H., Sanjad, S.A., Lifton, R.P. 1996. *Nat. Gen.* **14:**152–156
83. Soybel, D.I., Gullans, S.R., Maxwell, F., Delpire, E. 1995. *Am. J. Physiol.* **269:**C242–C249
84. Staley, K., Smith, R., Schaack, R., Wilcox, C., Jentsch, T.J. 1996. *Neuron* **17:**543–551
85. Stanton, B.A., Kaissling, B. 1989. *Am. J. Physiol* **257:**F1–F10
86. Sun, A., Grossman, E.B., Lombardi, M., Hebert, S.C. 1990. *J. Membrane Biol.* **120:**83–94
87. Take, C., Ikeda, K., Kurasawa, T., Kurokawa, K. 1991. *N. Engl. J. Med.* **324:**472–476
88. Tanimura, A., Kurihara, K., Reshkin, S.J., Turner, R.J. 1995. *J. Biol. Chem.* **270:**25252–25258
89. Tingley, W.G., Roche, K.W., Thompson, A.K., Huganir, R.L. 1993. *Nature* **364:**70–73
90. Tsukahara, H., Krivenko, Y., Moore, L.C., Goligorsky, M.S. 1994. *Am. J. Physiol.* **267:**F190–F195
91. Velazquez, H., Ellison, D.H., Wright, F.S. 1991. *Am. J. Physiol.* **253:**F555–F562
92. Verlander, J.W., Tran, T.M., Zhang, L., Kaplan, M.R., Hebert, S.C. 1996. *J. Am. Soc. Nephrol.* **7:**1293
93. Voss, T.G., Gatti, P.J., Fermin, C.D., Garry, R.F. 1996. *Virology* **219:**291–294
94. Wall, S.M., Trinh, H.N., Woodward, K.E. 1995. *Am. J. Physiol.* **38:**F536–F544
95. Xu, J.C., Lytle, C., Zhu, T.T., Payne, J.A., Benz, E., Jr., Forbush, B.3rd. 1994. *Proc. Natl. Acad. Sci. USA* **91:**2201–2205