 doi:10.20944/preprints202008.0457.v1

Role of the cation-chloride-cotransporters in cardiovascular disease

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Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 August 2020 doi:10.20944/preprints202008.0457.v1

Abstract

The SLC12 family of cation-chloride-cotransporters (CCCs), comprising potassium chloride cotransporters (KCCs)-mediated Cl^- extrusion relative to sodium chloride cotransporters (NKCCs)-mediated Cl^- loading, play vital roles in cell volume regulation and ion homeostasis. These functions of the CCCs influence a variety of physiological processes, many of which overlap with the pathophysiology of cardiovascular disease. Although not all of the cotransporters have been linked to Mendelian genetic disorders, recent studies have provided new insights into their functional role in vascular and renal cells along with their contribution to cardiovascular diseases. Particularly, an imbalance in potassium levels promote the pathogenesis of atherosclerosis and disturbances in sodium homeostasis are one of the causes of hypertension. Recent findings even suggest hypothalamic signalling as a key signalling pathway in the pathophysiology of hypertension. In this review, we summarize and discuss the role of CCCs in cardiovascular disease with particular emphasis on knowledge gained in recent years on NKCCs and KCCs.

Keywords: cardiovascular disease; hypertension; atherosclerosis; electroneutral transport; cation-chloride-cotransporters; KCCs; NKCCs

1 Evolution of the Cation Chloride Cotransporter Family

Comprehensive phylogenetic analysis by Hartmann et al revealed the existence of a single ancestral cation chloride cotransporter (CCC) gene in the Archean, *Methanosarcina acetivorans* [1]. The ancestral gene appears to be the base of numerous duplication events which led to paralogous CCC subfamilies in Archean and eukaryotes. These subfamilies consists of sodium chloride cotransporter (NCC), sodium potassium chloride co transporters (NKCCs, NKCC1 and NKCC2; with NCC collectively referred to as N[K]CCs), potassium chloride co transporters (KCCs, KCC1-4), polyamine transporter (CCC9) and CCC-interacting protein (CIP1). NCC and NKCCs are Na^+ driven, as they use Na^+ in their stoichiometric translocation of Cl^-, whereas KCCs are K^+ driven and uses K^+. CIP1 has been shown to inhibit NKCC1 activity [2] and enhance KCC2 [3] activity in cultured cells. CCC9 remains unclassified. CCCs provide electroneutral transport of sodium, potassium and chloride across the plasma membrane and are inhibited by several structurally similar compounds, such as bumetanide, furosemide (**Table 1**). Further gene-loss events resulted in the complex distribution of CCCs across the taxa. Interestingly, phenotypic analyses reveal that all plant CCCs belong to the KCC subfamily and that gene duplication events led to the formation of two distinct clades;

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Table 1. Major characteristics of Cl^--coupled cation cotransporters [4-7].

Gene	Human chromosome localization	Protein	Transported ions	Alternative spicing	Tissue distribution, cellular/subcellular expression	Link to disease	Inhibitors, IC50 (μM)
SLC12A2	5q23.3	NKCC1	Na^+, K^+, Cl^-	Isoforms A and B [8]	Ubiquitous: basolateral membrane of epithelial cells, non-epithelial cells	Schizophrenia [9]	Bumetanide, 0.05–0.60; Furosemide, 10–50; ARN23746 [10] Azosemide, 0.246-0.197
SLC12A1	15q21.1	NKCC2	Na^+, K^+, Cl^-	Isoforms A, B and F [11]	Kidney-specific: apical membrane of the thick ascending limb	Bartter's syndrome type I [12]	Bumetanide, 0.10–0.50; Furosemide, 15–60
SLC12A3	16q13	NCC	Na^+, Cl^-	NA	Kidney-specific: apical membrane of the distal convoluted tubule	Gitelman's syndrome [13]	Polythiazide, 0.5
SLC12A4	16q22	KCC1	K^+, Cl^-	NA	Ubiquitous	NA	Bumetanide, 60 [14]; Furosemide, 40 [14]; DIOA,~10
SLC12A5	20q13	KCC2	K^+, Cl^-	NA	Neurones	Epilepsy [15]	Bumetanide, 55; Furosemide, 10; VU 0463271, 0.061 [16]; DIOA,~10
SLC12A6	15q14	KCC3	K^+, Cl^-	Isoforms A and B [17]	Ubiquitous	ACCPN [18]	Bumetanide, 40; Furosemide, 25; DIOA,~10
SLC12A7	5p15	KCC4	K^+, Cl^-	NA	Ubiquitous	Renal tubular acidosis [19]	Bumetanide, 900; Furosemide, 900; DIOA,~10
SLC12A8	3q21	CCC9	NA	Six isoforms [20]	Ubiquitous	Psoriasis [20]	NA
SLC12A9	7q22	CIP1	NA	NA	Ubiquitous	NA	NA

NA, information not available.

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CCC1 and CCC2 [21]. Although knockout mutants in two plant species reported altered growth and development, functional disruption of plant CCC remain largely unknown due to limitations of existing heterologous expression systems [21]. Contrastingly, gene duplication within the CCC subfamilies of vertebrates resulted in subfunctionalization [1]. This process created a number of isoforms with different expression patterns and functionality in various organs and tissues, and have been extensively studied. Further variants are generated by alternative splicing of the isoforms with various ratio of expression in human tissues (**Table 1**).

Although the Na^+ driven and K^+ driven family reciprocally regulate Cl^-, uptake for N(K)CCs and extrusion for KCCs, the mechanism of coupled ion translocation is considered to be similar. In an alternating access model for NKCCs proposed by Haas, the carriers only move when they are either fully loaded or completely empty [22]. Following ions binding in a strictly ordered sequence, a conformation change occurs that causes the transporter to face the opposite direction and adopt a new form (inwards facing state during extracellular ion binding and vice versa) [22]. The ions are then released in the same order they were bound. Once empty, the transporters return to the original form ready for a new cycle. Recent 3D models have been made that support Haas, however, due to the many differences between paralogs of CCC family members, inferring functional properties from data obtained for a different family member may not be desirable [23].

The topology of all CCCs consist of 12 transmembrane domains (TMDs) and large intracellular amino and carboxyl terminal domains [23]. Notably, the K^+ driven and Na^+ driven subfamily vary in their position of the long extracellular loop: between TM5 and TM6 in KCCs and between TM7 and TM8 in the Na^+ driven subfamily. Within the isoforms of the subfamilies, the TMs and C-terminus are highly conserved. In contrast, the N terminus is much more variable among isoforms [23]. However, the TMs and not the termini are involved in the binding of diuretics [23], hence why loop diuretics like furosemide and bumetanide are frequently used to characterise the physiological function of KCCs and NKCCs with little specification for each paralog. Instead, the termini are involved in regulation of transport activity indicative of the sites for post translational modifications like phosphorylation that are located within them.

2 Functional regulation of the Cation Chloride Cotransporter Family

It is generally accepted that regulation of CCC function is accomplished through phosphorylation/dephosphorylation events. Phosphorylation/dephosphorylation reciprocally regulate the Na^+ and K^+ driven subfamily via a network of serine-threonine kinases and phosphatases [24-26]. Phosphorylation activates the Na^+ dependent subfamily and inactivates the K^+ dependent subfamily and vice versa [27]. The master regulator of CCCs, With-No-K

(Lysine) kinases (WNKs) regulate the CCCs via its downstream target, SPS1-related proline/alanine-rich kinase (SPAK) or the SPAK homolog oxidative stress-responsive kinase 1 (OSR1) [24-26]. This results in phosphorylation of threonine and serine residues of the CCCs. The number and position of phospho-sites vary between different family members as they are not evolutionary conserved among isoforms and paralogs [23]. Phosphatases such as protein phosphatase 1 (PP1) counterbalance the act of the kinases [28].

Recent data by Lee et al [29] challenges the current understanding of KCC regulation. Lee and team reported that phosphorylation of KCC2 specific residue, Ser940, by protein kinase C (PKC) have been shown to activate KCC2, an isoform exclusively expressed in the nervous system. However, the phosphorylation of highly conserved threonine residues in all KCCs are all associated with inactivation [26,30-32]. Thus the phosphorylation-mediated-activation of KCC2 may be an adaptive mechanism to assist with KCC2 functions in the nervous system [31,33,34]. Gene mutations in WNK1 and WNK4 result in over-activation of the WNK-SPAK/OSR1 pathway, increased phosphorylation and activation of NCC in the kidney, thus resulting in hypertension [35,36]. Recent studies highlight multiple other signalling factors and regulators of CCC activities that are implicated in cardiovascular disease [19,37-42]. More specifically, the functional regulation of CCCs in vascular smooth muscle cells and renal cells play a significant role in the pathogenesis of atherosclerosis and Mendelian and non-Mendelian forms of hypertension [19,39,43-45].

3 Role of NCC in cardiovascular disease

Previous studies have demonstrated that the thiazide-sensitive NCC in the distal convoluted tubule (DCT) plays an important role in blood pressure regulation and that loss of NCC function causes hypotension (**Figure 1**). Contrastingly, gain-of-function mutations that result in the over activation of NCC causes Gordon's syndrome, a Mendelian form of hypertension. Gordon's syndrome is caused by genetic mutations in genes encoding for the regulatory WNK-SPAK/OSR1 and its upstream regulator, an E3 ligase complex containing kelch-like 3 (KLHL3) and Cullin 3 (CUL3). Indeed, current research is focused on identifying novel targets within the WNK-SPAK/OSR1 pathway for use in Gordon's and potentially non-Mendelian forms of hypertension. A large body of evidence has demonstrated that inhibition of various components of the WNK-SPAK/OSR1-NCC pathway is an effective drug target for reducing blood pressure [46]. However, it has also been suggested that the lack of specificity across WNK and SPAK isoforms will mean that the drug discovery process will be challenging [39]. A more detailed account of the CUL3/KLHL3-WNK-SPAK/OSR1 pathway as a target for hypertension can be found in the review by Ferdaus et al [39].

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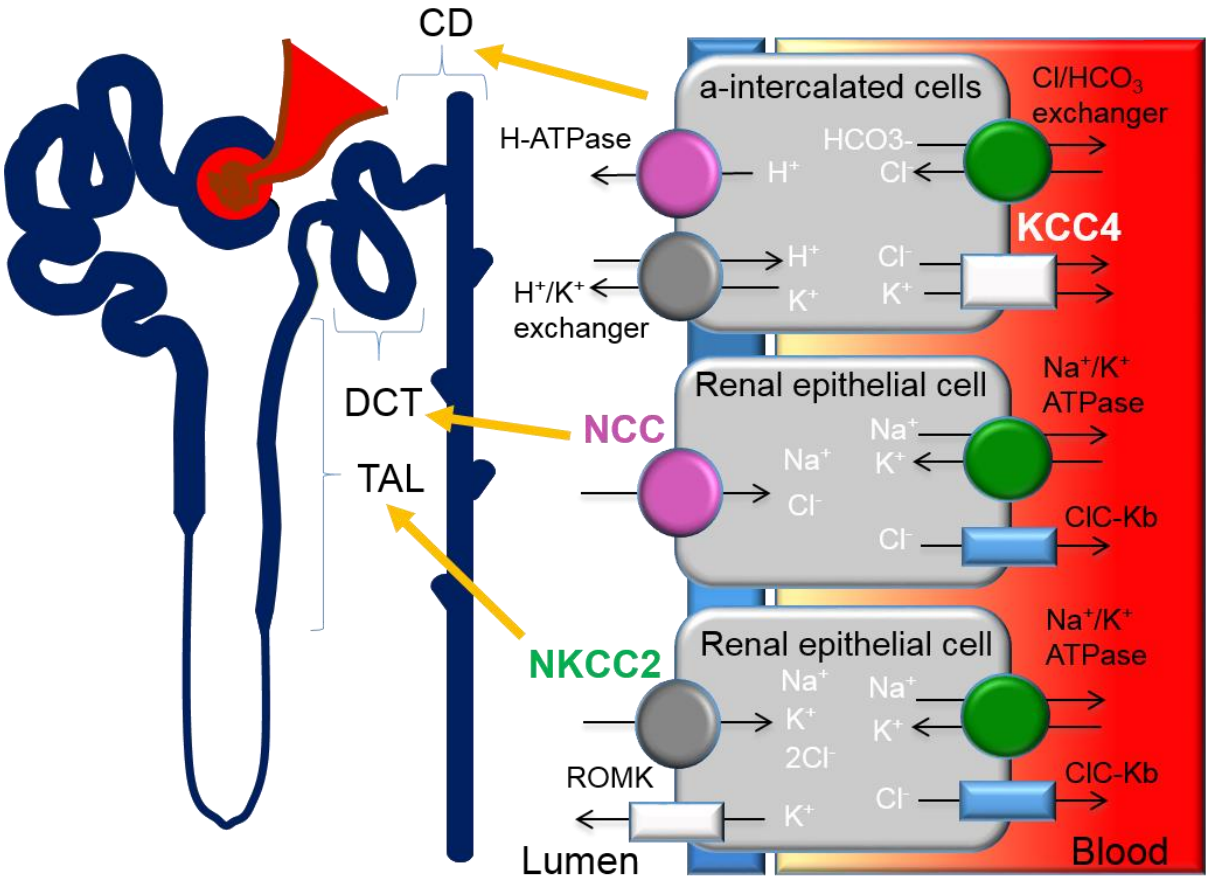

Figure 1. Expression of cation-chloride-cotransporters in the kidney. Alpha-intercalated cells in the collecting duct (CD) secretes acid via the apical H-ATPase and H^+/K^+ exchanger and reabsorbs bicarbonate via the basolateral Cl/HCO_3 exchanger. Efflux of Cl^- through the potassium chloride co-transporter-4 (KCC4) is important to maintain the electrochemical gradient to facilitate the acid secretion activities of the alpha-intercalated cells. Loss of KCC4 leads to renal tubular acidosis, which, if left untreated, could lead to cardiac arrhythmias. Sodium-chloride-cotransporter (NCC) are exclusively expressed in the distal convoluted tubule (DCT). Gain-of-function mutations in regulatory genes that lead to the over activation of NCC causes Gordon's syndrome.

4 Role of KCCs in cardiovascular disease

Loop diuretics, such as furosemide and bumetanide, are antihypertensive that acts primarily on NKCC2 in the thick ascending limb of the loop of henle (TAL) to inhibit reabsorption and consequently reduce blood pressure [47]. However, loop diuretics have also been shown to act on KCCs [48]. Although much of the work has been centred on understanding the role of KCC in vascular cells, a growing body of evidence indicates KCC participation in renal physiology [49,50]. Two isoforms, KCC3 and KCC4 are both differentially expressed in the kidney reflecting the variability of their role in cardiovascular disease; KCC3 may regulate blood pressure while KCC4 participates in maintaining the acid-base balance [49,50]. A secondary effect of loop diuretics is vasodilation. Several members of the KCC (KCC1-3-4) family have been identified in the vascular smooth muscle cells (VSMC) [48]. These evidence

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suggests a role for KCC in the VSMC. Researchers have reported that growth factors such as platelet derived growth factors (PDGF) [42] that promote and inhibitors such as nitric oxide (NO) [51] that inhibit phenotypic changes of the VSMC, are also modulators of KCCs in the VSMCs. Together with the observation of multiple cardiovascular defects in 3 models of KCC3 null mice [18,52,53], the evidence makes a compelling case for a pathogenic role of KCC beyond its well-studied physiological role in the erythrocytes. Here, we will discuss the substantial body of evidence suggesting a role of KCCs and their signalling pathways in the pathogenesis of cardiovascular disease.

4.1 KCC in vascular cells

Contractile VSMCs undergo phenotypic changes to proliferative/migratory cells in response to serum factors such as PDGF. Proliferative VSMCs in turn, forms lesions that are evident in vascular diseases such as atherosclerosis. Due to KCC participation in other proliferative diseases such as cancer [54] and reports of the functional significance of cell volume regulation in cell proliferation [55], a role for KCC in cell proliferation and migration is plausible. Indeed, Zhang and colleagues observed inhibition of KCC basal activity following a 24 hour serum deprivation and recovery upon serum addition [42]. Using synthetic VSMCs as a model system, the team found an increase in KCC1 and KCC3 activity after short term exposure (10 minutes) to PDGF and increased in KCC1 but decrease in KCC3 mRNA expression after long term exposure (12 hours) [42]. Consistent with evidence that KCC1 and KCC3 mRNA expression follows a 2:1 ratio in VSMCs [56], the results suggest that KCC1 may play a more vital role in PDGF-mediated modulation of VSMCs.

AG-1296, an inhibitor of the PDGF receptor, abolished the PDGF-induced increase in KCC activity, indicating PDGF activation of KCC through its membrane receptors [42]. Dimerization and auto phosphorylation of PDGF membrane receptors, initiates downstream signal transduction through molecules such as phosphatidyl-inositide-3-kinases (PI3-K) and mitogen-activated protein kinase (MAPK). Using pharmacological inhibitors, Zhang and team further concluded that PDGF-mediated activation of KCC is via the P13K/Akt pathway [57]. Although inhibition of MAPK by PD98059 have been shown to suppress KCC activity in red blood cells [58] and breast cancer cells [59], the researchers did not find evidence of MAPK involvement in PDGF activation by KCC. Notably, the study on breast cancer cells found that stimulation of KCC4 mRNA levels by insulin-like growth factor-1 (IGF-1), another serum growth factor, was inhibited by 65% with inhibitor of ERK1/2, PD98059, but was insensitive to the p38 MAPK inhibitor, SB202190 [59]. While inhibitor of Akt, LY 294002, only inhibited IGF-1 stimulation of KCC4 mRNA levels by 35% [59], suggesting ERK1/2 to be play a bigger role in regulation of KCC expression. However, it is important to note that the length of incubation

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vary between the experiments: 1 hour for VSMCs and 3 hours for red blood cells and breast cancer cells and that the importance of each signalling pathways could vary between cell types. The researchers also found that calyculin A, an inhibitor of protein phosphatase 1 (PP1), significantly inhibited PDGF regulation of KCC by 60% [57], suggesting the participation of PP1 in KCC modulation by the PDGF-PI3K pathway (**Figure 2**). As there is no clear evidence of PI3K regulation of PP1, further research will need to check if additional components downstream the PI3K exist.

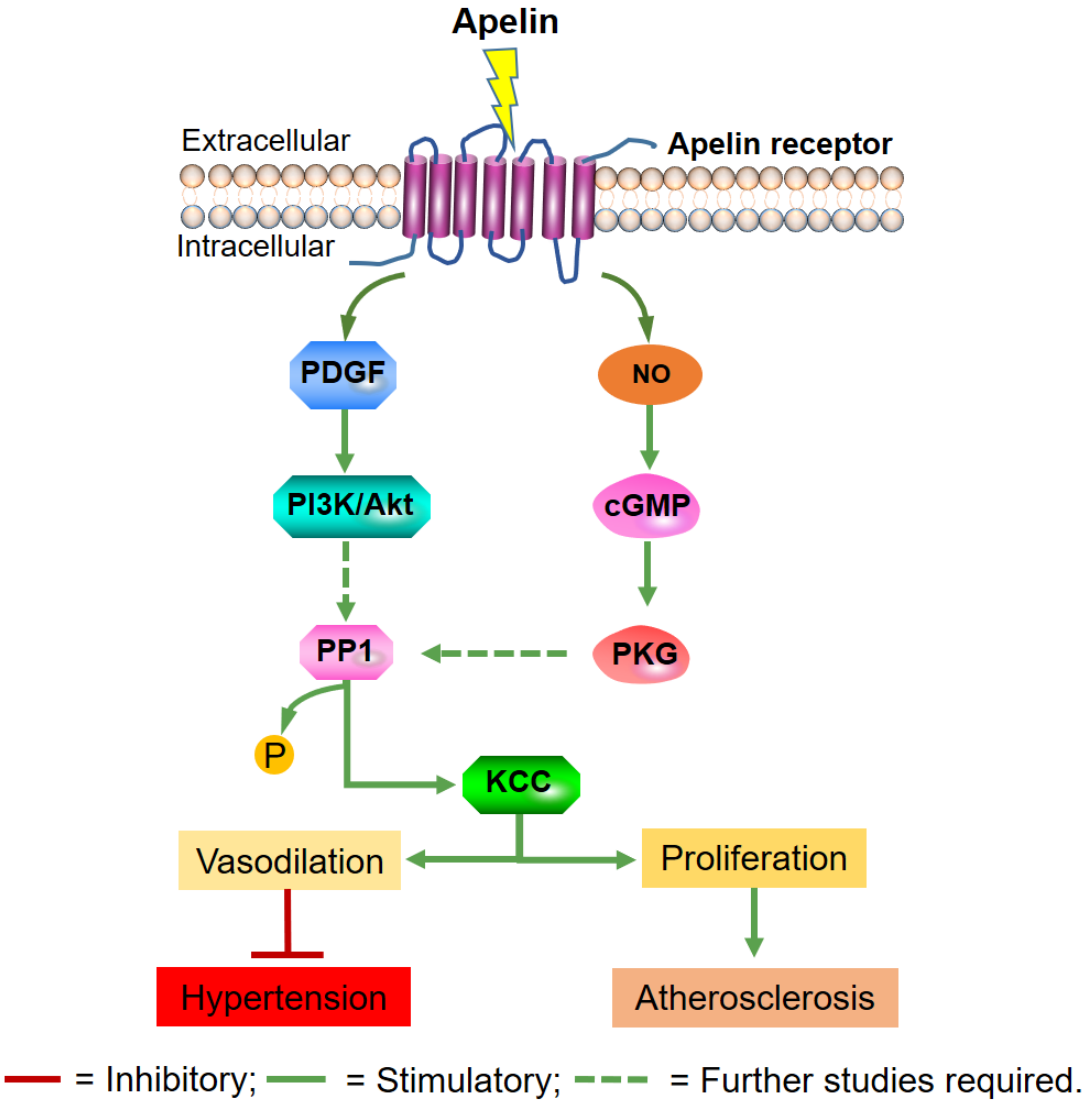

Figure 2. KCC is implicated in atherosclerosis and is a potential therapeutic target for hypertension. Potassium chloride channel (KCC) in vascular cells is regulated via the platelet-derived growth factor (PDGF) and the nitric oxide (NO), both of which are implicated in atherosclerosis and vasodilation respectively. PDGF regulates KCC through phosphoinositide 3-kinase (PI3K). Through an unknown mechanism, PI3K activates protein phosphatase 1 (PP1) which dephosphorylates and actives KCC. Activation of KCC could enhances the phenotypic switching of vascular smooth muscle cells (VSMC) into a diseased state. NO regulates KCC via the nitric oxide/ cyclic guanosine monophosphate /protein kinase G (NO/cGMP/PKG) pathway. PKG activates PP1 through an unknown mechanism leading to

KCC activation and consequent vasodilation. KCC could be a potential therapeutic target for hypertension. Apelin is cardioprotective and a common modulator of both pathways.

Although the evidence is clear that PDGF regulate KCC, it is not clear how KCC activation plays a role in PDGF-mediated signalling to promote atherosclerosis. Recent kinetic studies of KCC activity found evidence that support for direct KCC involvement in vascular phenotypic changes [44]. The researchers found significant changes in the kinetic parameters for the co-transport of Cl^- between early, intermediate and late VSMC synthetic phenotypes. Increased KCC1 and KCC4 expression in the late synthetic VSMC in comparison to earlier states suggest a functional role of KCC in phenotyping switching to a diseased state.

Nitric oxide acts to regulate the function of VSMCs via cGMP dependent activation of protein kinase G (PKG) to promote VSMC relaxation. Activators of the NO/cGMP/PKG pathway such as sodium nitroprusside, NONOates (NO donors) and 8Br-cGMP (PKG substrate) have been shown to stimulate KCC activity, mRNA and protein expression while inhibitors such as KT5823 (PKG inhibitor), calyculin A and genistein (PP1 inhibitor) inhibit KCC activity, mRNA and protein expression [37]. Researchers also found that when the vasodilators hydralazine and sodium nitroprusside were added to pre-contracted arteries, the arteries relax despite the blocking of all pathways except for KCC [37]. Interestingly, the NO signalling preferentially increased KCC3b mRNA by 8.1-fold in comparison to 2.5-fold for KCC3a isoform despite a 3:1 KCC3a:KCC3b mRNA expression [60]. Overall, this indicates KCC contribution to the vascular effects of these vasodilators. Studies by Di Fulvio and team reported NO/cGMP/PKG signalling pathway and PKG participation in the regulation of KCC1 [51] and KCC3 [56] mRNA expression respectively. Further studies by Adragna et al found that baseline KCC activity was higher in PKG transfected cells (PKG+) in comparison to PKG deficient cells (PKG-) [61]. Furthermore, deletion of KCC3 isoform has been shown to cause severe hypertension in mice [62]. These findings suggest that KCC may be involved in vasodilation possibly through the NO/cGMP/PKG-PP1 pathway (**Figure 2**).

A common modulator of both the PI3K/AKT and the NO/cGMP/PKG signalling cascades in the VSMC is Apelin [63]. Apelin stimulates the NO pathway to induce vasodilation and the PI3K/Akt pathway to induce proliferation and migration of VSMCs [64]. Due to its cardioprotective effects in the VSMC's, Apelin has been suggested as a novel therapeutic target in the cardiovascular system [64]. In 2013, researchers found that Apelin stimulated KCC activity through the NO pathway by 336% and 142% by the MAPK/PI3K pathway [65]. In contrast, oxidized plasma cholesterol (oxLDL), a known promoter of vascular lesions, inhibited KCC by 70% and treatment with Apelin restored this function [65]. Although these studies prove that KCC3 has a physiological role in the vascular cells and thus have potential as

therapeutic targets, it is not clear if KCC acts through modifying the membrane potential, changes in cell volume or influence of intracellular chloride activity.

A study by Rust et al reported an increase in $[Cl^-]_i$ but no difference in vascular contractility in KCC3 knockout mice [66]. Instead, pharmacological inhibition of the sympathetic nervous system reduces blood pressure after 80 seconds and produced an increase in urinary excretion of catecholamine. Additionally, the isolated arteries from the KCC3 null mice did not response differently to vasoactive interventions in comparison to wild type (WT) mice [66]. This suggests that KCC3 inactivation likely contributes to hypertension through neurogenic mechanisms and that the increase in $[Cl^-]_i$ did not significantly affect vascular contractility. However, recent studies by Garneau et al found normal levels of circulating catecholamine, supporting a role for KCC3 in the vasculature [67]. It is important to note that although both groups studied contractile properties, the study by Rust used isolated saphenous arteries while Garneau et al used thoracic aortas. Furthermore, the isolated saphenous arteries used by Rust were hypertrophied and presented with increased $[Cl^-]_i$ compared to the WT littermates. Thus more than one mechanisms could be a contributing factor to the cardiovascular phenotype.

Taken together, these studies identified KCC as a key mediator of vascular pathologies. KCCs are implicated in atherosclerosis through its regulation by PDGF and hypertension via regulatory NO (**Figure 2**). It is important to note that although the functional role of each KCC isoform remains obscure due to the lack of specific inhibitors for each KCC isoform, the comprehensive effects of KCC on VSMCs proliferation is evident.

4.2 KCC in renal cells

Three isoforms, KCC1, KCC3 and KCC4 were found to be expressed in the kidney [68]. Although KCC1 is ubiquitous, its expression has only been studied at the mRNA level. Thus the role of KCC1 in the kidney remains unknown. KCC3 is exclusively expressed in the basolateral membrane in the proximal tubule and KCC4 is expressed in the basolateral membrane of proximal tubules, thick ascending limb of loop of Henle (TAL) and in alpha intercalated cells in the connecting duct. KCC has been shown to be elevated in patients (n=4) suffering from Liddle syndrome, an inherited form of high blood pressure [69], suggesting a role of KCC in blood pressure regulation. Melo et al evaluated the expression level and distribution of KCC3 and KCC4 in rats exposed to hyperglycaemia, a low-salt diet, metabolic acidosis and low or high K^+ diet [49]. Consistent with previous discovery of glucose stimulation of basolateral efflux of K^+ ions [70], Melo et al found that KCC3 mRNA and protein expression were increased during hyperglycaemia but not with low-salt diet or acidosis. In contrast, KCC4

protein expression was increased 1.53±0.16 fold by a low sodium diet (n=5, P<0.05) in the kidney and metabolic acidosis (n=5, P<0.05) specifically in the alpha-intercalated cells of the collecting duct (CD) [49]. Although the results suggest that KCC3 is only involved in glucose reabsorption mechanism, the upregulated KCC4 expression in low-salt diet and metabolic acidosis implicates KCC4 in salt reabsorption of the TAL and acid secretion in the CD. More specifically, the increase in KCC4 activity during salt restriction could be a compensatory mechanism to promote basolateral K^+ efflux to support salt transport. The results from Melo is consistent with previous studies by Boettger et al who reported renal tubular acidosis in KCC4 deficient mice [19]. Boettger reported compensatory metabolic acidosis along with increased urine alkalinity in WNK4 knockout mice (pH7.3±0.1) in comparison to the WT littermates (pH 6.4 ± 0.1) (n=7, P <0.001) [19]. Energy-dispersive X-ray microanalysis revealed increased $[Cl^-]_i$ in alpha-intercalated cells of KCC4 knockout mice. The alpha-intercalated cells in the kidney secretes acid via the apical H-ATPase and H^+/K^+ exchanger and reabsorbs bicarbonate via the basolateral Cl^-/HCO_3^- exchanger (**Figure 1**) [50]. The rise in $[Cl^-]_i$ indicates a more alkaline intracellular pH and electrochemical imbalance which inhibits apical H^+ secretion, resulting in type 1 renal tubular acidosis. Cl^- extrusion through KCC4 is important to negate the influx through the Cl^-/HCO_3^- exchanger. Cl^- import and bicarbonate extrusion through the Cl^-/HCO_3^- compensates for the acid secretion on the apical side. Thus, KCC4 play a physiological role in renal acidification. Loss of KCC4 leads to renal tubular acidosis, which presents with hypokalaemia. If untreated, cardiac arrhythmias can occur due to the low level of K^+ in the blood.

5 Role of NKCC in cardiovascular disease

As previously mentioned, loop diuretics such as furosemide and bumetanide are antihypertensive that inhibit sodium reabsorption in the kidney and consequently reduce blood pressure [47]. Loop diuretics bind to the TM of CCCs, a region that is highly conserved, and thus are not specific to one subfamily or their isoforms [23]. As such, pharmacological studies have revealed key roles for the ubiquitous NKCC1 and renal specific NKCC2 in vascular and renal cells respectively. NKCC1 are involved in the suppression of myogenic response in microcirculatory beds [45,71] and NKCC2 are involved in blood pressure regulation through sodium reabsorption.

5.1 NKCC in vascular cells

Myogenic tone is the ability of blood vessels to constrict. Constriction of vessels, elevates peripheral resistance, consequently raising blood pressure. Pharmacological inhibition with bumetanide, a potent inhibitor of both isoforms of NKCC, was found to suppress and block myogenic tone in mouse mesenteric arteries and rat afferent arterioles respectively [72].

However, the inhibitory action of bumetanide on myogenic tone and contractions are absent in NKCC1 null mice. This indicates that HCDs influence myogenic tone via NKCC1 and that NKCC1 could have hypotensive effects in vascular cells. This is further supported by Meyer et al who reported that tail-cuff measurements of blood pressure in NKCC1 deficient mice was significantly ($P<0.01$) lower (114.5±12.2mmHg, n=16) than the WT mice (131.8±2.5 mmHg, n=16) [73]. Although previous studies using the same measurement method found no statically significant reduction in blood pressure in NKCC1 null mice (n=5, P=0.54) [74], the larger number of mice used by Meyer et al and the longer length of time period (21 days) is conclusive evidence that NKCC1 is implicated in the maintenance of vascular tone. However, it is important to consider that although mice lacking NKCC1 have reduced blood pressure, the contractile effect of NKCC1 does not necessarily translate into the effects of blood pressure *in vivo.*

Further studies by Garg et al found that bumetanide is less efficient in hypertension due to a fixed aortic coarctation, a model for fixed increase in resistance in the aorta, in comparison to hypertension by continuous infusion of norepinephrine [75]. Although it is unclear if blood pressure regulates NKCC1 or vice versa, it can be concluded that the hypotensive actions of bumetanide is through systemic vascular resistance rather than cardiac output and that NKCC1 influences blood pressure through the smooth muscle tone in resistance vessels. Like all muscle cells, VSMC uses the rise in intracellular [Ca^{2+}] concentration ([$Ca^{2+}]_i$) as a trigger for contraction. Thus myogenic tone is dependent on the influx of calcium [Ca^{2+}] into VSMCs via voltage-dependent Ca^{2+} channels and intracellular stores. Cl- channel activation leads to Cl- efflux, which depolarises the membrane. This activates the voltage dependent Ca^{2+} channel and elevates [$Ca^{2+}]_i$. The rise in [$Ca^{2+}]_i$ mediates myosin-actin interaction, cross bridge cycling and consequently, VSMC contraction [76]. In the VSMC, NKCC1 is responsible for the inward directed flux of Cl^-. Consistent with the observation of reduced blood pressure in NKCC1 null mice, inhibition of NKCC1 by bumetanide decreased [Cl^-], Ca^{2+} uptake and completely blocked contraction [77]. This observation coupled with reports of lack of effect by bumetanide on contraction in response to KCl, which depolarizes smooth muscle cells directly, suggests that NKCC1 contributes to vascular tone via maintenance of [$Cl^-]_i$. Further studies have proved that this is accomplished through the channel opening of NKCC1 which increases the [$Cl^-]_i$ above the electrochemical equilibrium which depolarizes the VSMC through activation of voltage-gated Ca^{2+} [78]. Consistent with previous findings, more recent studies in intact isolated thoracic aortas demonstrated that NKCC1 is involved in phenylephrine-induced rhythmic contraction in the mouse aorta and that NKCC1 is regulated by calcium sparks [79].

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A study by O'Donnell et al showed that NKCC1 activity of the VSMC from spontaneously hypotensive rats is significantly less in comparison to normotensive rats [80]. Evidence also showed that NKCC1 activity is upregulated in different models of hypertension and is accompanied by an increase in $[Cl^-]_i$ [81]. These models include hypertension elicited by deoxycorticosterone (DOCA)/salt and angiotensin 2 they showed that these caused increased levels of NKCC1 mRNA. And aldosterone increases NKCC1 activity. Although it is clear that NKCC1 is implicated in the maintenance of vascular tone, NKCC1 upregulation in hypertensive models raises the question whether NKCC1 activity and upregulation increases blood pressure or is the result of hypertension. Using rat aorta, Jiang et al showed that after aortic coarctation, a procedure that yields both hypertensive and hypotensive segments of the aorta in the same animal, the NKCC1 activity of the hypertensive segment of the aorta is 62% greater than the control. There was also a 21% decrease in NKCC1 activity in the hypotensive segment of the aorta [82]. This is accompanied by observations of a fivefold increase in NKCC1 mRNA in the hypertensive aorta compared with the hypotensive or normotensive aorta. This supports the theory that blood pressure regulates NKCC1 in VSMC.

A study by Lee et al found that expression of NKCC1 mRNA and protein levels in the aorta and heart tissues are higher in spontaneous hypertensive rats (SHR) than in the normotensive rats [45]. Lee also found greater hypomethylated NKCC1 gene promoter in the aorta and heart of the SHR relative to the normotensive rats ($p<0.01$). This was accompanied by increased NKCC1 expression and inhibitory action of bumetanide on mesenteric artery contractions with age in SHR but not wild type. The Emax for the dose response curve was 74±2.3 for SHR and 102±4.7 for normotensive rats. This suggests that promoter hypomethylation upregulates NKCC1 in SHR. Lee concluded that upregulation of NKCC1 could be responsible for the development of high blood pressure in SHR. If this is true, NKCC1 promoter hypomethylation could be a marker of hypertension development. Further research by the same group found that the expression of NKCC1 is epigenetically regulated during postnatal development of hypertension [83]. Sequencing revealed that the NKCC1 promoter in wild type mice were increasingly methylated with age (8.5%, n=5) but remained largely hypomethylated in SHR (2.2%, n=5) during postnatal development of hypertension. The activity of DNA methyl transferase 3B (DNMT3B), a family of enzyme that transfer methyl group to promoter regions to downregulate their expression, is also 3 fold higher in the aorta of wild type in comparison to SHR at 18 weeks of age. This suggest that the maintenance of hypomethylation in NKCC1 promoter as a result of decreased DNTB3B activity is the reason for age-dependent development of hypertension in SHR. In another study, Lee et al also noted that inhibition of DNMT with an inhibitor in the rat cerebral cortex results in the upregulation of the transcription of NKCC1 during postnatal maturation [40]. Another research group reported that DNMT

inhibition also resulted in an increase of blood pressure [84]. Contrary to previous findings, these results could mean that age and blood pressure alter NKCC1 promoter epigenetically. However, more studies will need to be completed in vascular cells to further elucidate the relationship between blood pressure and NKCC1 methylation.

Further studies also found that NKCC1 is upregulated via histone modification in the aortas of angiotensin II (ang-II) induced hypertensive rats. Cho et al found that the level of NKCC1 mRNA and protein in the aortas increased gradually in the ang-II infused rats [85]. A histone activating code, acetylated histone H3, was increased and histone repressive code, trimethylated histone H3, was reduced in ang-II infused rats compared to the sham. Studies have shown that the recruitment of specificity protein 1 by CpG hypomethylation leads to the upregulation of NKCC1 in hypertensive rats [71]. This could mean that NKCC1 is epigenetically upregulated by histone modification ($P<0.05$) or DNA demethylation upon the development of hypertension ($P<0.01$) and that the methylation of CpG dinucleotides may play a role in the epigenetic maintenance of blood pressure through decreased expression of NKCC1. These findings further emphasizes NKCC1 methylation or histone modification as a potential biomarker in the diagnosis and management of hypertension. More recently, studies by Ji [86] and Andersen [87] et al found increased NKCC activity after cardioplegia induced arrest of diabetic hearts ($n=5$, $P<0.001$) and in post infarction heart failure ($n=6$-7) respectively. These findings indicate the potential role of NKCC in protection of the diabetic heart after cardioplegia infusions and remodelling after myocardial infarction.

These studies collectively suggest that inhibition of NKCC1, specifically in the smooth muscle could be a pharmacological target for hypertension. However, the ubiquitous nature of NKCC1 coupled with the possibility of concurrent inhibition of NKCC2 in TAL, would preclude currently available NKCC1 inhibitors to be used in the treatment of cardiovascular disease. This is due to the large doses required to achieve an inhibitory response in the plasma which would produce unacceptable toxicity elsewhere [88]. Thus, a compound that selectively inhibits NKCC1 over NKCC2 and is not excreted via the urine is desirable. Indeed, Savardi et al.2020 has now discovered a new drug candidate, termed ARN23746, which active selectively for NKCC1 *in vivo* versus NKCC2 [10].

5.2 NKCC in renal cells

Despite kidney specific expression of NKCC2, NKCC1 has been found in several locations in the kidney including the inner medullary collecting duct. The basolateral expression of NKCC1 results in salt and water secretion whereas apical expression of NKCC2 in the thick ascending limb of the Loop of Henle results in salt and water reabsorption (**Figure 1**). Mice without

NKCC1 reported impaired epithelial chloride secretion [72] and loss-of-function mutations of NKCC2 results in the salt wasting phenotype of type 1 Bartter Syndrome [89]. The secretory NKCC1 is also upregulated in metabolic acidosis [90]. An extensive account of the role of NKCC1 and NKCC2 in hypertension can be found in a recent review by Orlov et al [91].

6 Hypothalamic signalling mechanisms in hypertension

The hypothalamus is crucial for the central control of blood pressure in response to central and peripheral stimuli. As such, there has been many advances implicating the hypothalamic signalling mechanisms in hypertension. For the purpose of this review, we will only focus on the signals that directly involve the CCCs. Those are the hypothalamic neuronal signalling mechanisms and the hypothalamic regulation of vasopressin secretion. A complete review of hypothalamic signalling mechanisms in hypertension can be found here [43].

Accumulating evidence implicates increased sympathetic drive from the paraventricular nucleus (PVN) in the development of hypertension. More specifically, the hyperactivity of the PVN contributes to the elevated sympathetic drive. The excitability of the PVN is regulated by excitatory glutamergic and inhibitory GABAergic inputs. Although GABA is the main inhibitory neurotransmitter in the brain, there is evidence that GABA can also elicit excitatory response. Polarity of the neurotransmitter depends on the GABA equilibrium potential (E_{GABA}). A more positive E_{GABA} results in excitatory signalling and a more negative results in inhibitory signalling. The E_{GABA} is influenced by $[Cl^-]_i$. If the $[Cl^-]_i$ is high, the E_{GABA} will be more positive and the GABA signalling will result in depolarization. If $[Cl^-]_i$ is low, E_{GABA} will be more negative and the response is an inhibitory and result in hyperpolarization. The Cl^- in neurons are determined by two cation-chloride cotransporters, NKCC2 and KCC2. Thus a disruption of Cl^- homeostasis leads to impairment of balance between inhibitory and excitatory signals in the PVN.

In spontaneous hypertensive rats (SHR), GABAergic inhibition in the PVN is impaired [92]. Studies by Ye et al revealed that the E_{GABA} undergoes a depolarizing shift in SHR ($n=9$, $p > 0.05$) [41] . Inhibition of NKCC1 ($n=7$, $p < 0.001$) normalizes the E_{GABA} and restores GABA inhibition of PVN in SHRs [41]. The mRNA and protein levels of NKCC1 but not KCC2 are significantly increased in SHRs and that the inhibition of NKCC1 significantly reduces the sympathetic vasomotor tone ($n=9$) [41]. This study provides evidence that NKCC1 activity is important for chloride homeostasis in the PVN. A disruption of the homeostasis, diminishes the inhibitory effects of GABA resulting in the increased sympathetic outflow observed in hypertension. This is supported by studies in mouse model of genetically hypertensive mice [93].

Arginine vasopressin (AVP) is secreted by magnocellular neurosecretory cells (MNC) in the PVN. AVP stimulates water reabsorption in the kidney to maintain blood pressure. In the development of hypertension, AVP neuronal activity is dysregulated. Studies by Yi et al found an increased in AVP expression in the AVP of SHR [94] and that this change increases with both age and magnitude of hypertension. This is due to reduced GABAergic input from baroreceptors in response to high salt intake. Further findings by Kim et al found that the inhibitory to excitatory switch of GABA receptors in AVP neurones contribute to the increasing AVP released observed in hypertension [95]. The switch is driven by upregulation of NKCC1 and downregulation of KCC2. Recently, findings suggest that the downregulation of KCC is mediated by brain-derived neurotrophic factor via the tyrosine kinase receptor B (TrkB) pathway [38]. The downregulation of KCC prevents the inhibitory GABAergic signalling evoked by the baroreceptor which leads to increased excitability of AVP secreting neurons. The increased excitability stimulate excess AVP release consequently increasing blood pressure [38].

Viewed collectively, these studies provide insight into the regulation of sympathetic activity and AVP secretion in normotensive and hypertensive phenotypes *in vivo*. Sympathetic activity is increased in hypertensive phenotype due to disruption in chloride homeostasis following an upregulation of NKCC1 and AVP secretion is increased due to downregulation of KCC2. This suggest that further investigation of these mechanisms may result in multiple therapeutic targets to reduce sympathetic activity and AVP release in hypertension.

6 Role of regulatory WNK-SPAK/OSR1 pathway in cardiovascular disease

In 2001, research in the Lifton lab reported that mutations in WNK1 and WNK4 genes caused a Mendelian form of hypertension, pseudohypoaldosteronism type 2 or Gordon's syndrome [35]. Further research identified a missense mutation in WNK4 (D561A) to cause the hypertension in Gordon's syndrome via activation of the WNK-OSR1/SPAK-NCC cascade [96]. This is supported by the observation that SPAK deficiency rescues Gordon's syndrome caused by WNK4 mutation [97]. WNK4 has even been found to inhibit NCC expression through activating the MAP/ERK pathway [98]. Although homozygous WNK1 knockout mice is embryonic lethal, studies of WNK1 heterozygous mice revealed a significant decrease ($n=11$, $p<0.002$) in blood pressure [99]. However, a study by Susa et al found that the blood pressure in WNK1 heterozygous mice was not reduced even when fed a low salt diet ($n=8$, $p=0.101$). The team also did not find a significant decrease in the phosphorylation of OSR1, SPAK, NCC, NKCC1 and NKCC2 in the kidney [100]. In contrast, a significant decrease in the phosphorylation of NKCC1 in the aorta and decreased pressure-induced myogenic response

in the mesenteric arteries was observed in WNK1 heterozygous knockout mice. This is further supported by studies completed by the Bergaya team [101] who did not find a decrease in basal systolic blood pressure despite the use of radiotelemetry, a method widely recognised as the best for establishing blood pressure phenotype [102]. Consistent with Susa et al, Bergaya et al also found a major loss of contractile myogenic response in WNK1 heterozygous mice that are associated with decreased phosphorylation level of WNK1 substrate SPAK and its target NKCC1 in arteries (n=16, p=0.00005). These studies confirm the contribution of NKCC1 in the regulation of blood pressure and suggests a role for WNK1 in the regulation of NKCC1. Further studies in WNK1 knockout mice revealed that WNK1 is needed for angiogenesis and heart development [103]. SPAK knockout mice presents with Gitelman syndrome and impaired vasoconstriction [104]. Thus SPAK has been suggested as an important pharmacological target for the treatment of essential hypertension [105]. More recent studies have shown that the upstream regulator of WNK, KLHL3 and CUL3 is implicated in Gordon's syndrome. Particularly, mutations in CUL3 causes severe hypertension by affecting both renal and vascular function [106,107]. More specifically, mice with mutant CUL3 protein showed increased expression of RhoA, a molecule involved in regulation of vascular tone [107]. Further information on the mechanisms of CUL3/KLHL3 pathogenesis in Gordon's syndrome can be found in a review by Ferdaus et al [108].

We recently employed studies of phosphoproteomics and functional kinomics and found that WNKs regulate SPAK/OSR1, facilitating the phosphorylation of KCCs, which consequently reduces KCC activity, and the dephosphorylation of NKCC1, increasing NKCC1 function [26,32]. Thus, the same kinase pathway produces inverse effects on the opposing co-transporters, providing a powerful push-pull regulatory control of $[Cl^-]_i$. Through the use of *in vivo* SPAK mouse model, we then found a role for SPAK or OSR1 as a bridge to facilitate the signalling cascade between WNKs and CCCs [109]. Furthermore, we have developed a novel SPAK binding inhibitor, termed ZT-1a, which specifically blocks the WNK-SPAK/OSR1-CCC signalling pathway, subsequently reducing the NKCC1 and KCCs phosphorylation in cultured cells, and *in vivo* mouse and rat tissues [110]. This is promising for the treatment of cardiovascular disease as ZT-1a may interfere with the SPAK regulation of $[Cl^-]_i$ homeostasis via NKCC1 and KCCs in cardiac cells.

7 Conclusion

Together, the review highlights the potential role of CCCs in cardiovascular disease. Gain of function mutations of WNKs resulting in the enhanced activity of NCC in the kidney is the cause of Gordon's syndrome. Increase in KCC activity in vascular cells could lead to atherosclerosis and have potential vasodilation effects. Impairment of KCCs in renal cells

contribute to renal acidosis, a disease if not treated could lead to cardiac arrhythmias. NKCC1 is epigenetically upregulated in hypertension. Impairment of NKCC2 in renal cells lead to Bartter's syndrome. CCCs are also involved in the hypothalamic signalling in hypertension and the mutations in the regulatory CUL3/KLHL3-WNK-SPAK/OSR1 pathway contributes to the pathology of Gordon's syndrome. The future development of interventional strategies should exploit these recent findings pertaining the role of CCCs in vascular and renal cells as well as the pathophysiology mechanisms involved in cardiovascular disease.

Box 1 Measuring KCC3 and NKCC1 activity Ion-Transport Activity

The cation chloride cotransporters (CCC) are electroneutral thus electrophysiology methods that use voltage changes and electrical current cannot be used to measure CCC activity. Instead, researchers exploit the characteristic difference of the intracellular concentration of the ions to measure their activity instead. The most commonly used method is unidirectional Rb^+ flux analysis to measure cotransporter activity [111]. The method uses non-radioactive Rubidium ($^{86}Rb^+$) as a potassium congener and measures the flux of $^{86}Rb^+$. In order to characterize the transporter itself, all other transporters will need to be blocked. For example, when studying KCC3 activity in VSMCs, oubain and bumetanide were used to block Na^+/K^+ pump and NKCC1 activities. This method was used to clone and characterize the function of NKCCs [112] and function of KCC3 [113]. More importantly, the technique allows for the study of channel properties in a variety of conditions including homogenous cell preparations (erythrocytes, oocytes and different heterologous cell lines. However, it is difficult to use in brain slices and neuronal culture. Alternatively, fluorescent assays to measure movement of thallium ions through potassium channels are also used [114]. This method uses Tl^+ as a surrogate of K^+ and a Tl^+ sensitive fluorescent dye (FlixORTM) to visualize Tl^+ uptake through channels in single cells. The Delpire group reported similar results between $^{86}Rb^+$ and FluxOR [115]. Other techniques include the ammonium pulse technique which uses cardiac strips to measure acidification rate [116]. A comparative analysis of measuring cation-chloride-cotransporter activity can be found in a review by Medina et al [111].

Acknowledgements

This study was in part supported by the University of Exeter Medical School start-up fund (J.Z.).

Additional information

Conflicts of Interest: The author declares no conflict of interest.

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