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Regulation Of Nkcc2 Trafficking By Vesicle Fusion Proteins Vamp2 And Vamp3 In The Thick Ascending Limb

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REGULATION OF NKCC2 TRAFFICKING BY VESICLE FUSION PROTEINS VAMP2 AND VAMP3 IN THE THICK ASCENDING LIMB

by

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Advisor Date

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DEDICATION

To my parents Ana and Carlos for their unconditional love and support.

To my family and friends for their constant encouragement.

To the memory of my grandparents, undying source of inspiration.

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I would like to thank my advisor and mentor Dr. Pablo Ortiz for his guidance and encouragement. His valuable advice and his commitment in my formation always pointed me in the right direction. I couldn't have been more fortunate to find a role model with such enthusiasm and passion for science.

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PREFACE

"Come forth into the light of things, let nature be your teacher" – William Wordsworth.

The dissertation that follows is the result of my training as a graduate student at the laboratory of Dr. Pablo Ortiz at the Hypertension and Vascular Research Division in the Henry Ford Hospital, Detroit. During this time we tried to understand a fundamental molecular process that controls sodium absorption by specialized renal cells. The seminal work that led to this dissertation was a research article published by Dr. Ortiz in 2006 before I joined his laboratory. In that work he determined that two proteins, VAMP2 and VAMP3, regulate the presence of the renal co-transporter NKCC2 at the plasma membrane of renal cells. An important conclusion from that work was that trafficking of NKCC2 to the cell membrane is a critical mechanism to regulate NaCl absorption by the kidney, a process with profound consequences in arterial pressure. It became evident that understanding how NKCC2 reaches the plasma membrane will provide valuable insights into the mechanisms by which the kidneys maintain fluid and electrolyte balance and blood pressure. By the time I joined the team, the focus of the research was clearly pointing in that direction.

In the years preceding the preparation of this dissertation, we published four original articles all aimed to characterize the mechanisms that control NKCC2 trafficking under physiological and pathological conditions. That research led to the hypothesis tested in this dissertation. Here we demonstrate that VAMP2 and VAMP3 mediate two different pathways in NKCC2 trafficking. Inhibition of these pathways for NKCC2 delivery to the cell surface alters renal function and decreases blood pressure. The data used in this thesis are in the process of being published. We have submitted two manuscripts to the *Journal of*

Biological Chemistry. They are titled: a) “Vesicle-Associated Membrane Protein 2 (VAMP2) but not VAMP3 Mediates cAMP-Stimulated Trafficking of the Renal Na-K-2Cl Co-transporter NKCC2 in Thick Ascending Limbs”, and b) “Vesicle-Associated Membrane Protein 3 (VAMP3) Mediates Constitutive Trafficking of the Renal Na-K-2Cl Co-transporter NKCC2 in Thick Ascending Limbs: Role in Renal Function and Blood Pressure”.

CHAPTER 1

BACKGROUND AND GENERAL HYPOTHESIS

The kidneys and control of blood pressure

The functional units of the kidneys are tubular structures called nephrons (Figure 1). In each nephron, blood is filtered in the glomerulus into the tubular space in Bowman's capsule. The filtrate is then modified along the nephron to form urine, which is delivered to the renal pelvis, collected in the bladder and finally excreted. Along the nephron we find specialized segments with different absorptive and secretory properties. One of these segments is the loop of Henle, which penetrates deep into the renal medulla. The distal portion of the ascending loop of Henle is the thick ascending limb (TAL), which is divided in a medullary and a cortical portion. The main function of the TAL is to absorb ions that contribute to the creation of a hyperosmotic interstitium. The resulting osmotic gradient in the medullary interstitium drives water absorption in the collecting duct, concentrating the urine.

The production of urine by the kidneys finally results in elimination of waste

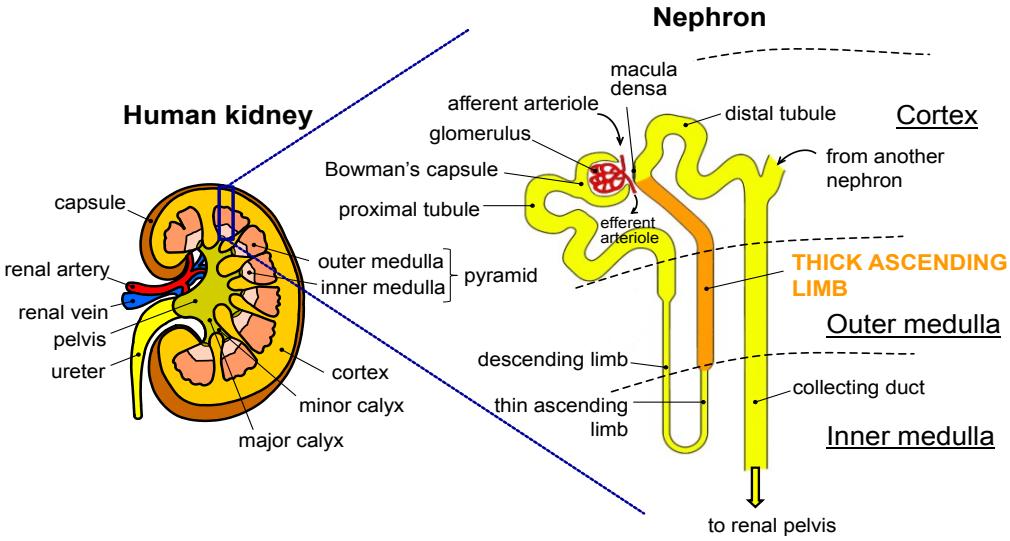

Figure 1: Representation of the general kidney anatomy and organization of the nephron.

metabolites and excretion of ions and osmolytes, which regulate blood *p*H, osmolality and water content. In addition, the kidneys also secrete vasoactive agents. The combination of these processes allows tight regulation of fluid volume in extracellular compartments and short- and long-term control of blood pressure.

NaCl absorption by the thick ascending limb: role of NKCC2

The TAL reabsorbs 25-30% of the NaCl that was filtered from the blood at the glomerulus. The main function of the TAL is to dilute the forming urine by absorbing Na, Cl and Ca while water absorption is close to zero. This creates a hyperosmotic interstitium *via* a countercurrent multiplier mechanism that provides the osmotic force to absorb water from the collecting duct. In this way, the TAL influences the ability of the kidney to reabsorb water and regulate arterial pressure.

The currently accepted model for NaCl absorption by the TAL is depicted in Figure 2. The epithelial cells defining the TAL walls exhibit apical-basolateral polarity. In the basolateral membrane, the Na-K ATPase provides the driving force by generating an electrochemical gradient (1-3). This electrochemical gradient is used by the apical Na/K/2Cl co-transporter NKCC2 and the Na-H exchanger NHE3 (4,5) to absorb Na, K and Cl from the lumen. Na leaves the cell though the basolateral Na-K ATPase and Cl exits through Cl channels and K/2Cl co-transporters (3,6,7). Potassium is

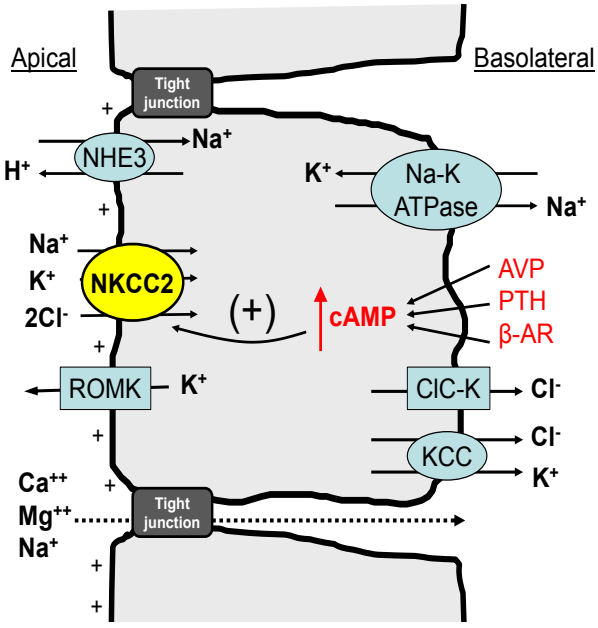

Figure 2: Model for NaCl absorption by the thick ascending limb.

recycled back to the tubular lumen *via* the renal outer medullary potassium channel ROMK (8). The accumulation of positive charges in the luminal membrane due to ROMK creates a lumen-positive electric potential that drives Na, Ca and Mg absorption through the paracellular pathway (9,10).

About 80% of the Na and 100% of the Cl reabsorbed by the TAL enter the cell *via* NKCC2, which is expressed exclusively in an apical location in the TAL and the macula densa (11-13). Activation of cAMP signaling in the TAL is a potent stimulus for NKCC2 and NaCl absorption (14-19). Conversely, NKCC2 inhibitors like the loop diuretic furosemide (20) and intracellular messengers like nitric oxide (20-22) and cGMP (15,23,24) decrease NaCl absorption by the TAL. NKCC2-mediated NaCl absorption by the TAL is crucial in regulation of blood pressure (25-30).

Role of NKCC2 in control of blood pressure

Loss of function mutations in NKCC2 cause Bartter syndrome type I (27,31-36), a condition characterized by polyuria, inability to concentrate urine, loss of Na, Ca and Mg in the urine and low blood pressure (37-39), all symptoms associated with impaired TAL function. Consistent with this, genetic disruption of the NKCC2 gene in mice produces a Bartter-like phenotype (40). Both Bartter syndrome patients and NKCC2 knockout mice experience severe dehydration at an early age that can be life-threatening unless fluid balance is restored. Two independent studies (41,42) have shown that rare NKCC2 mutations in the human population cause reduced NKCC2 activity and decrease blood pressure.

Opposite to loss-of-function NKCC2 mutations, enhanced NKCC2-mediated NaCl absorption by the TAL has been linked to salt-sensitive and spontaneous hypertension in animal models (43-46) and has been associated with salt sensitivity in humans (25,47), in

particular the African-American population (48).

NKCC2 is also the target site of the loop diuretics bumetanide, furosemide and torasemide, which decrease blood pressure (20,49-55). However, they are not routinely used in the long term treatment of hypertension due to side effects from the profound volume and electrolyte depletion. This highlights the important contribution of NKCC2 to maintaining fluid homeostasis.

Role of cAMP in NKCC2 function and NaCl absorption

Hormonal stimulation of cAMP by vasopressin (2,56-61), calcitonin (57,62-64), parathyroid hormone (PTH) (56,63,65) or β-adrenergic receptors (66-69) enhances NKCC2-mediated NaCl absorption in the TAL. The stimulatory effect of cAMP on NKCC2-dependent NaCl absorption is mainly mediated by protein kinase A (PKA) (17,18,70,71). This kinase directly phosphorylates NKCC2 at Serine 126 in the amino-terminus and Serine 874 in the carboxy-terminus (residues in the NKCC2 rat sequence) (72). Mutagenesis analysis indicate that elimination of Ser-126 decreases NKCC2 activity *in vitro* (73).

Two other phosphorylation sites at the amino terminus of NKCC2 become phosphorylated indirectly after stimulation with vasopressin (74), threonines 96 and 101 (rat sequence) and are also required for normal NKCC2-mediated ion transport *in vitro* (75). However, the signaling pathway that leads to Thr-96 and Thr-101 phosphorylation by vasopressin is not clear.

Altogether, these pieces of evidence indicate that the cAMP-PKA pathway is an important stimulus for NKCC2 and TAL activity. In this dissertation we studied the molecular mechanism that mediates cAMP stimulation of NKCC2 in TALs and how it differs from the mechanism that controls NKCC2 under basal non-stimulated conditions.

Regulation of NKCC2 by protein trafficking

In order to transport ions from the lumen to the intracellular space, NKCC2 has to be inserted in the apical membrane. However, most NKCC2 is found in sub-apical vesicles in the TAL as observed by electron microscopy (76). Our laboratory has shown that under baseline conditions only 3-5% of total NKCC2 is expressed at a steady-state in the cell surface (14). Despite this small proportion, a decrease in surface NKCC2 expression lowers NaCl reabsorption by the TAL to a great extent (14,24). Steady-state surface NKCC2 levels are the result of exocytic delivery (77) and endocytic retrieval (78,79). We will refer to this process as protein trafficking (Figure 3). Exocytic delivery can occur from the biosynthetic pathway and from recycling compartments at the same time. Previous data from our laboratory indicate that only a small fraction of internalized NKCC2 undergoes recycling back to the plasma membrane (78).

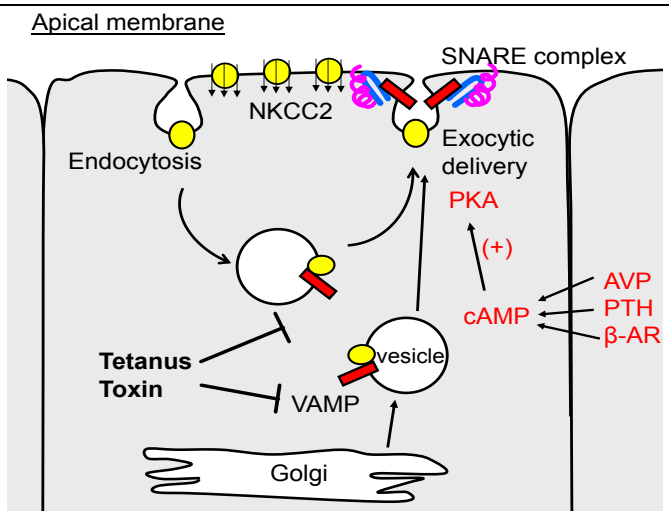

Figure 3: NKCC2 trafficking to the apical membrane in the thick ascending limb.

NKCC2 is present at the cell surface under baseline conditions (14,24,77), indicating that trafficking occurs constitutively in the absence of stimulation. However, cAMP stimulates steady-state surface NKCC2. This can be the result of either stimulated exocytic delivery or decreased endocytic retrieval. We have found that vasopressin stimulates exocytic delivery of NKCC2 in TALs (74,77,80). Stimulation of β-adrenergic receptors also augments surface NKCC2 *via* cAMP (69). Inhibition of PKA blunts cAMP-stimulated exocytic delivery and cAMP-stimulated steady-state surface NKCC2 expression (77).

In addition, we have shown that surface NKCC2 was abnormally high in a rat model of salt-sensitive hypertension (46). Altogether, these observations illustrate the tight relationship between NKCC2 presence at the apical surface and NaCl absorption by the TAL. This also highlights the importance of NKCC2 trafficking as a mechanism to regulate renal function and blood pressure.

The SNARE family of membrane fusion proteins

In order to reach the apical membrane, NKCC2 has to be delivered from intracellular vesicles to the plasma membrane. The details of this mechanism are largely unknown. In other eukaryotic cells, vesicles deliver their cargo by means of membrane fusion. This process is mediated by the family of proteins known as Soluble NSF Attachment protein REceptors (SNARE) (81-84). There are three types of SNAREs: Vesicle-Associated Membrane Proteins (VAMP), syntaxins and Synaptosome-Associated Proteins (SNAP). VAMPs reside in the vesicle membrane (85,86) and syntaxin and SNAP are associated with the target membrane (87-89). When the vesicle approaches the target membrane, the three SNAREs combine with each other and form a helical complex (90,91) that drives fusion of the vesicle membrane with the target membrane (82,92-96). This is the basis of the SNARE hypothesis and is accepted to be universal for all eukaryotic cells (83,84).

There are seven VAMP isoforms, at least fifteen syntaxin isoforms and four SNAP isoforms (97-99). In addition, SNAREs bind to accessory proteins to complete the membrane fusion process (100-104). The different combinations of SNAREs and accessory proteins help direct protein trafficking along different cell compartments in a specific way according to the cell type and under defined physiological conditions.

Role of SNAREs in trafficking of renal transporters

In the kidney, SNAREs are probably best characterized in the collecting duct, where

they mediate cAMP-stimulated trafficking of the water channel aquaporin-2, the H-ATPase and the epithelial Na channel ENaC (105-120). For instance, VAMP8 knockout mice exhibit impaired translocation of aquaporin-2 in the collecting duct and fail to concentrate urine (121). In the TAL, expression of SNAP-23 (122), syntaxin 3 and 4 (118,123,124), and VAMP2 and 3 (14) has been reported. However, their role in NKCC2 trafficking remains largely unexplored.

Tetanus toxin selectively cleaves VAMP2 and VAMP3. Our laboratory has found that tetanus toxin prevents the stimulatory effect of cAMP on surface NKCC2 expression and NaCl absorption (14), indicating that VAMPs mediate cAMP-stimulated NKCC2 trafficking. However, tetanus toxin does not distinguish between VAMP2 and VAMP3. In addition, the details of the mechanism that mediates NKCC2 trafficking remain unknown. In this dissertation we addressed the relative contribution of VAMP2 and VAMP3 to the process of apical NKCC2 trafficking in the TAL.

Role of VAMP2 and VAMP3 in trafficking of transmembrane transporters

In epithelial cells, SNAREs exhibit polarized distribution, mediating distinct membrane fusion processes at specific cell compartments (123,125-136). Only the VAMP isoforms 2, 3, 7 and 8 have been reported to participate in exocytic processes in polarized epithelia (106,113,121,128,137-144). However, VAMP7 (145) and VAMP8 (146) traditionally mediate homotypic fusion between intracellular vesicles. In renal epithelia including the TAL and the collecting duct, VAMP2 and VAMP3 localize apically (14,147). Cleavage of VAMP2 and VAMP3 with tetanus toxin (which does not cleave VAMP7 and VAMP8) inhibits cAMP-stimulated NKCC2 trafficking in the TAL (14). However, it is not clear whether VAMP2 and VAMP3 mediate different pathways that control constitutive or cAMP-stimulated NKCC2 exocytic delivery.

In other cells, VAMP3 has been associated with early endosomes and may be involved in recycling (148-150), whereas VAMP2 has been reported to be excluded from recycling compartments (151). This suggests distinct compartmentalization of the two isoforms, at least in some cases. VAMP2 has been shown to mediate cAMP-stimulated delivery of transmembrane proteins (106,113,116,137,139,152). In the collecting duct, VAMP2 co-localizes with aquaporin-2 in the same intracellular vesicles (137,152) and is required for cAMP-stimulated translocation to the apical membrane (106,113). In addition, in other cells VAMP2 is regulated by phosphorylation by ATM kinase (153) and PKC (154,155). In this dissertation we proposed that VAMP2 selectively mediates cAMP-stimulated NKCC2 exocytic delivery whereas VAMP3 mediates constitutive delivery.

Protein-protein interactions as a possible mechanism for VAMP-mediated exocytosis

There are some examples in the literature of protein-protein interactions between SNAREs and transmembrane proteins (87,156,157). VAMP2 for instance, interacts with P/Q-type calcium channels (158) and the potassium channel Kv2.1 (159,160) in neurons. These interactions may be of physiological relevance since VAMP2- Kv2.1 interaction facilitates Kv2.1 inactivation (159,160). In the collecting duct, VAMP2 interacts with the proton pump H-ATPase and tetanus toxin prevents H-ATPase exocytic insertion (116). In the TAL, VAMP2 co-localizes with NKCC2 (14) and VAMP3 is also located apically. However whether they interact with NKCC2 is unknown.

It is known that protein-protein interactions regulate NKCC2. The Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1) kinase interact with NKCC2 *in vitro* (161). These kinases phosphorylate the amino-terminus of NKCC2 (162). The myelin and lymphocytes-associated protein (MAL) also interacts with NKCC2

(163) and decreases NKCC2 retrieval from the plasma membrane in cultured cells. Moesin, a member of the ezrin-radixin-moesin family, interacts with the carboxy-terminus of NKCC2 and is necessary for NKCC2 delivery to the plasma membrane (164). Two additional NKCC2 interactors include Aldolase B (165) and the secretory carrier membrane protein 2 (SCAMP2) (166). Both of them decrease NKCC2 at the cell surface in heterologous expression systems. Aldolase B stimulates NKCC2 retrieval from the membrane (165) and SCAMP2 inhibits exocytic delivery (166). In this dissertation, we tested whether VAMP2 and VAMP3 interact with NKCC2 as a possible mechanism that mediates NKCC2 exocytic delivery.

General hypothesis and project aims

In this work we tested the general hypothesis that **VAMP2 and VAMP3 interact with NKCC2 in the thick ascending limb and differentially mediate constitutive and cAMP-stimulated exocytic delivery of NKCC2 to the plasma membrane.** We addressed this hypothesis in the four aims that follow and are illustrated in Figure 4.

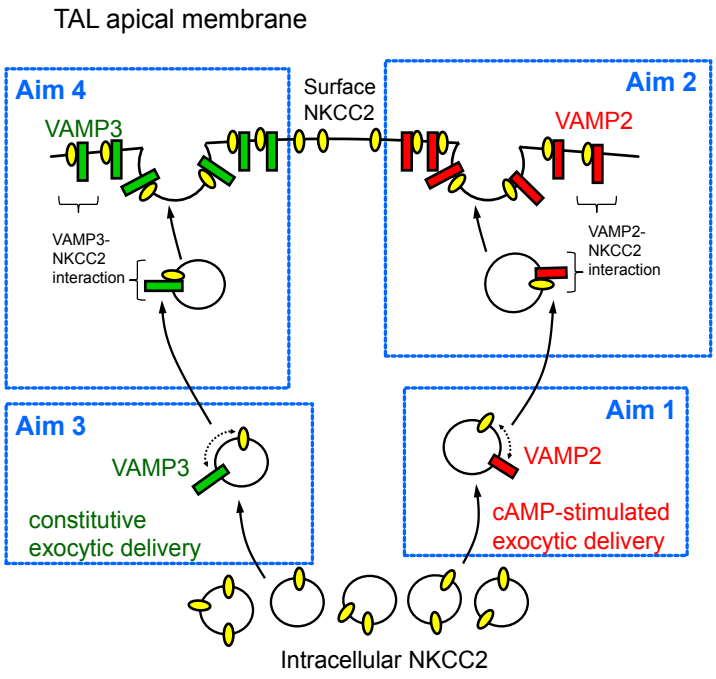

Figure 4: General hypothesis and project aims.

Aim 1- Hypothesis: VAMP2 mediates cAMP-stimulated trafficking of NKCC2 in the thick ascending limb.

In order to differentiate the role of VAMP2 from VAMP3, we silenced VAMP2 *in vivo*

with short hairpin RNAs (shRNAs) in rat TALs. Then we tested the effect of silencing VAMP2 on constitutive and cAMP-stimulated NKCC2 trafficking and NKCC2 expression.

Aim 2- Hypothesis: cAMP stimulates NKCC2-VAMP2 interaction and co-localization in thick ascending limbs.

We addressed the possibility that VAMP2 may mediate cAMP-stimulated NKCC2 trafficking by protein-protein interactions. We measured VAMP2 co-immunoprecipitation with NKCC2 in TALs and identified the region of NKCC2 that interacts with VAMP2. We measured co-localization of NKCC2 and VAMP2 at the apical surface of the TAL. We tested whether cAMP increases delivery of VAMP2 to the apical membrane of TALs and whether it enhances NKCC2-VAMP2 interaction.

Aim 3- Hypothesis: VAMP3 mediates constitutive trafficking of NKCC2 in the thick ascending limb.

We used shRNAs to silence VAMP3 *in vivo* in TALs and tested whether VAMP3 mediates constitutive or cAMP-stimulated NKCC2 trafficking. Finally, we obtained TALs from VAMP3 knockout mice to measure whether total and steady-state surface NKCC2 expression was decreased.

Aim 4- Hypothesis: VAMP3 interacts with NKCC2 in the thick ascending limb and maintains normal renal function and blood pressure.

To study whether VAMP3 interacts with NKCC2 we performed VAMP3-NKCC2 co-immunoprecipitations in TALs. We also measured co-localization of NKCC2 and VAMP3 at the apical surface of the TAL. Finally, to correlate VAMP3 dysfunction with renal function, we measured urine excretion and arterial pressure in VAMP3 knockout mice.

CHAPTER 2

ROLE OF VAMP2 IN cAMP-STIMULATED NKCC2 TRAFFICKING IN THE THICK ASCENDING LIMB

Introduction

Biological pathways that stimulate cAMP production, such as hormonal stimulation with vasopressin or β-adrenergic receptors, enhance NaCl reabsorption by the TAL by increasing steady-state surface NKCC2 expression (14,74,77,80,167). We have previously shown that cAMP increases steady-state surface NKCC2 by stimulating exocytic delivery of the co-transporter to the TAL surface (77). However, the molecular mechanism by which cAMP stimulates NKCC2 trafficking in TALs remains uncharacterized. In this chapter we tested whether VAMP2 mediates cAMP-stimulated NKCC2 trafficking, since VAMP2 mediates cAMP-stimulated exocytosis in other cells (106,113,168,169). We also explored whether the underlying mechanism may involve protein-protein interactions between NKCC2 and VAMP2. We tested this in two aims, one addressing the effect of silencing VAMP2 on NKCC2 trafficking and the other one focused on NKCC2-VAMP2 interaction and how it is affected by cAMP. We will discuss the relevance of NKCC2-VAMP2 interaction to NKCC2 trafficking and activity.

Aim 1- Hypothesis: VAMP2 mediates cAMP-stimulated trafficking of NKCC2 in the thick ascending limb.

Rationale

Our laboratory has previously shown that VAMP2 and VAMP3 are expressed in the TAL (14). However, a more complete profile of the VAMP isoforms expressed in the TAL is still missing. We began by screening other VAMPs previously shown to be expressed in

polarized epithelia, i.e. VAMP7 and VAMP8 (106,113,121,128,137-144).

We previously showed that tetanus toxin blocks the stimulation of cAMP on NKCC2-mediated NaCl absorption (14). Since the toxin does not distinguish between VAMP2 and VAMP3, in this aim we used silencing shRNAs *in vivo* to address whether VAMP2 mediates cAMP-stimulated NKCC2 exocytic delivery. In non-renal cells, VAMP2 has frequently been associated with stimulated exocytosis (151,170-172). Also, VAMP2 mediates cAMP-stimulated exocytosis of synaptic vesicles in neurons (168) and cAMP-stimulated delivery of the water channel aquaporin-2 in the renal collecting duct (106,113). In addition, in renal juxtaglomerular cells, VAMP2 mediates cAMP-stimulated secretion of renin (169). Interestingly, VAMP3 does not mediate cAMP-stimulated renin release. Although most of this evidence suggests that VAMP2 mediates cAMP-stimulated exocytic delivery of NKCC2 in the TAL, this has never been directly addressed. In this aim we tested whether VAMP2 mediates cAMP-stimulated NKCC2 trafficking. In order to increase intracellular cAMP, in our experiments we used forskolin to stimulate cAMP production by adenylyl cyclases, and IMBX to inhibit cAMP degradation by phosphodiesterases. We have used this maneuver in the past to produce a potent stimulation of NKCC2 trafficking in TALs (14,77).

A reliable biochemical methodology to study NKCC2 trafficking is by means of surface biotinylation, which will be explained in detail later. We have used this technique in the past in medullary TAL suspensions in combination with Western blot to specifically detect NKCC2 at the TAL surface (14,24,77). The specificity of the technique is achieved by using a specific anti-NKCC2 antibody directed against the amino-terminus of NKCC2 (173). The amino-terminal region is highly divergent from the closely related NKCC1 co-transporter (174), which is expressed in basolateral membrane of the collecting duct and

could be contaminating our TAL suspensions. In this way, if there is minimal contamination with other nephron segments in our preparations, any confounding effect is minimized by detecting specifically NKCC2, which is expressed exclusively in the TAL.

Results

Expression of VAMP isoforms in the thick ascending limb

We detected VAMP2 and VAMP3 expression in TAL lysates by Western blot, consistent with our previous report (14). In addition, we also observed single bands of the expected molecular weight for VAMP7 and VAMP8 (n = 4) (Figure 5). This was the first evidence that these other isoforms are expressed in the TAL.

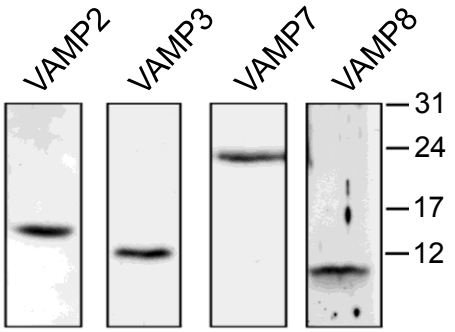

Figure 5: Expression of VAMPs in the thick ascending limb.

Developing of short-hairpin RNAs for VAMP2 silencing

To differentiate the effects of tetanus toxin that may be mediated by VAMP2 but not VAMP3, we developed silencing shRNAs to specifically decrease VAMP2 expression. First we designed small interference RNA (siRNA) sequences targeting a region of the rat VAMP2 transcript that does not overlap with other VAMP isoforms. Then we synthesized nude siRNAs and tested them in the cell line NRK-52E derived from rat kidney. Once silencing efficiency was confirmed by Western blot (Figure 6a), we designed shRNAs and

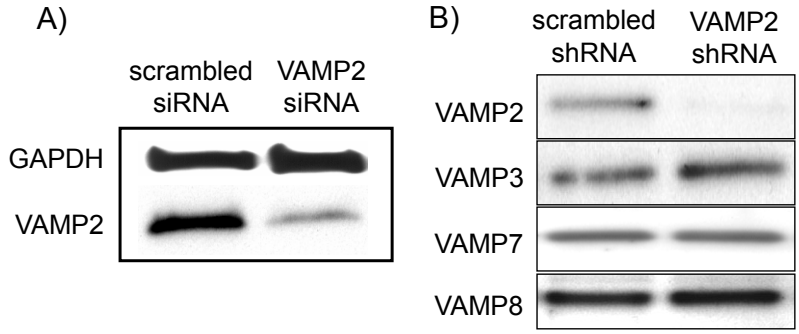

Figure 6: VAMP2 silencing *in vitro.* A) VAMP2 silencing *via* siRNA in NRK-52E cells. B) VAMP2 silencing *via* adenovirus-delivered shRNA in NRK-52E cells.

produced adenovirus particles. The efficiency of the adenoviruses was tested again *in vitro* by transducing the rat cell line. We observed a high degree of VAMP2 silencing that did not decrease expression of the other VAMPs (Figure 6b).

In vivo silencing of VAMP2

We delivered the shRNAs *in vivo* to rat TALs *via* injections in the outer medulla as shown in Figure 7. We exposed the kidneys *via* a small incision in the flank and administered five injections with calibrated needles in order to reach the outer medulla. Previous work from our laboratory demonstrated that injections with a syringe pump at a rate of 20 µl/minute would assure correct administration of the adenoviruses (175). To determine whether *in vivo* silencing was successful, we obtained TAL suspensions from the outer medulla of injected kidneys and measured VAMP2 expression by Western blot at different days. Figure 8a shows the decrease in VAMP2 expression we observed after 3 days of silencing. This is when we observed maximum decrease in VAMP2 expression, which was 69 ± 7% (p<0.01; n = 3) compared with TALs from the

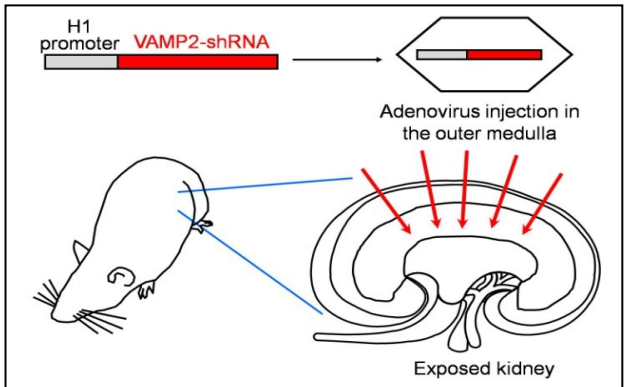

Figure 7: *In vivo* delivery of adenoviruses to the outer medulla of rat kidneys.

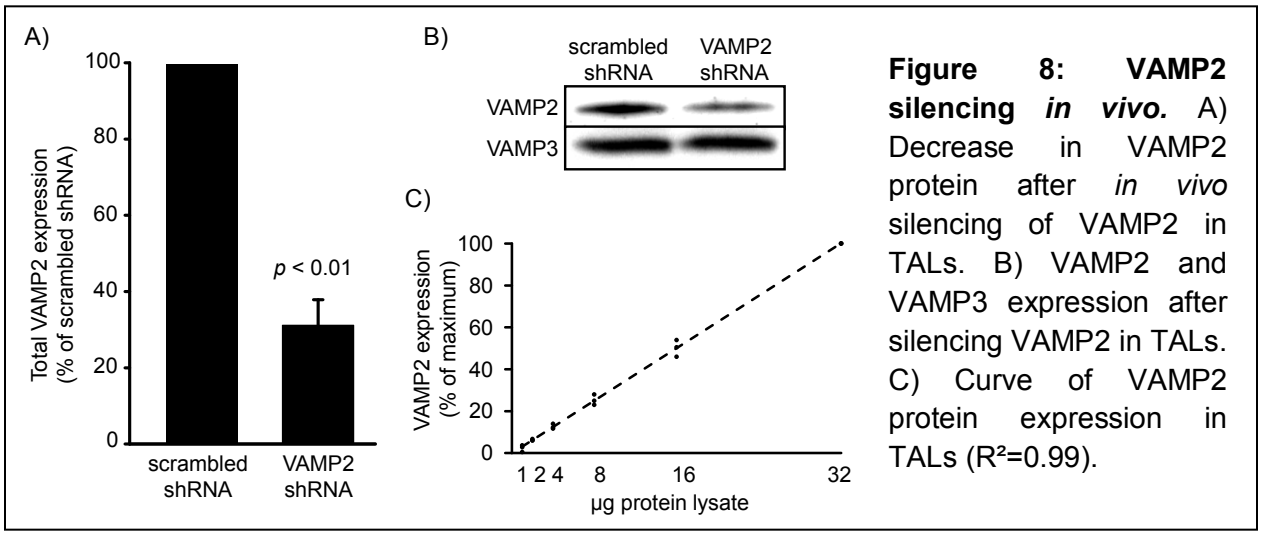

Figure 8: VAMP2 silencing *in vivo*. A) Decrease in VAMP2 protein after *in vivo* silencing of VAMP2 in TALs. B) VAMP2 and VAMP3 expression after silencing VAMP2 in TALs. C) Curve of VAMP2 protein expression in TALs (R^2=0.99).

contra lateral kidney injected with control scrambled-shRNA adenoviruses. VAMP2 silencing did not affect VAMP3 expression *in vivo* (Figure 8b), indicating that the sequence retained specificity. To assure that we would be able to detect changes in VAMP2 expression, we used 10 µg of protein in the Western blots. This amount of protein falls within the linear range shown in Figure 8c. Altogether these data indicate that the procedure for silencing VAMP2 *in vivo* is efficient and specific.

Effect of VAMP2 silencing on steady-state surface NKCC2

To test whether silencing VAMP2 decreases cAMP-stimulated NKCC2 trafficking, we measured steady-state surface NKCC2 expression by surface biotinylation in TALs treated with vehicle or forskolin + IBMX to stimulate cAMP production. We performed the treatment at 37ºC because we needed the TALs to reach a steady-state in surface protein expression. However, for surface

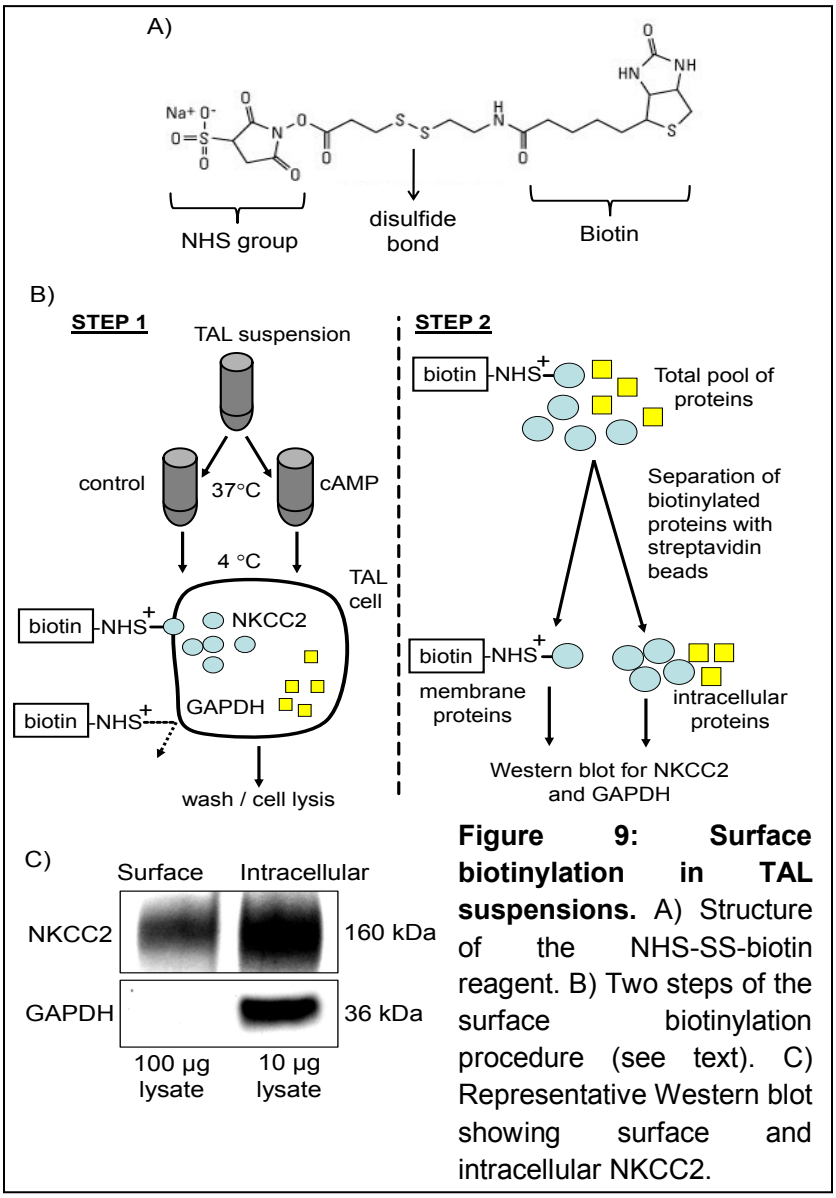

Figure 9: Surface biotinylation in TAL suspensions. A) Structure of the NHS-SS-biotin reagent. B) Two steps of the surface biotinylation procedure (see text). C) Representative Western blot showing surface and intracellular NKCC2.

biotinylation we needed to stop protein trafficking rapidly. We achieved this by quickly cooling the TAL suspensions to 4ºC. Then we proceeded with the surface biotinylation. We incubated the TALs with a biotinylation reagent consisting on a biotin portion linked *via* a disulfide bond to an NHS group (Figure 9a). The NHS group reacts with extracellular lysines in surface proteins (Figure 9b). Since the biotinylation reagent is charged, it does not cross the plasma membrane, therefore it does not biotinylate intracellular proteins. In this way, when lysing the TAL suspensions, we obtained a total pool of proteins consisting on surface, biotinylated proteins and intracellular, non-biotinylated proteins. We separated the biotinylated proteins by pull down with streptavidin-conjugated agarose beads and recovered them by boiling in reducing conditions to break the disulfide bond. NKCC2 was detected at the surface and in the intracellular fraction by Western blot (Figure 9c). The intracellular protein GAPDH was used a control since it was not detected at the surface.

To test the effect of VAMP2 silencing, we obtained TALs from VAMP2-shRNA transduced rat kidneys and measured constitutive and cAMP-stimulated steady-state surface NKCC2 (Figure 10). We observed that VAMP2 silencing did not affect constitutive

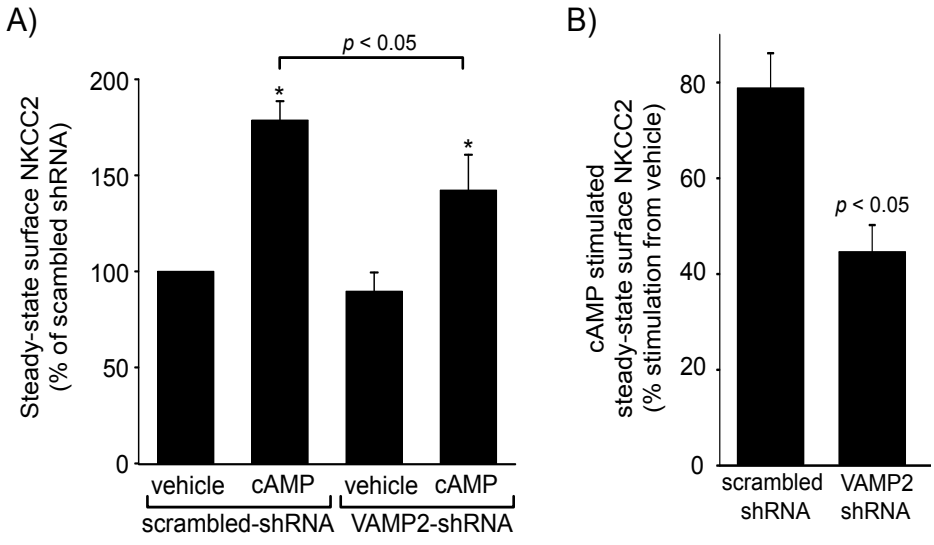

Figure 10: VAMP2 silencing decreased cAMP-stimulated but not constitutive steady-state surface NKCC2 in TALs. A) Steady-state surface NKCC2 in scrambled- and VAMP2-shRNA transduced TALs. *p<0.05 *vs.* vehicle. B) cAMP-stimulated steady-state surface NKCC2 in TALs.

steady-state surface NKCC2 expression (scrambled-shRNA: 100% *vs.* VAMP2-shRNA: 91 ± 8%; n = 7). However, cAMP stimulated steady-state surface NKCC2 by 79 ± 7% in control TALs, but in VAMP2-shRNA transduced TALs stimulation was only 45 ± 6% (p<0.05 *vs.* scrambled-shRNA) (Figure 10a). The quantification of the per cent increase after cAMP stimulation is shown in Figure 10b. Silencing VAMP2 blunted the stimulatory effect of cAMP on steady-state surface NKCC2 by 43% (p<0.05).

These data indicate that VAMP2 mediates cAMP-stimulated but not constitutive steady- state surface NKCC2 expression in the TAL.

Effect of VAMP2 silencing on NKCC2 exocytic delivery

The decrease in cAMP-stimulated steady-state surface NKCC2 we observed when we silenced VAMP2 may be due to reduced NKCC2 delivery to the surface. We next tested whether VAMP2 mediates cAMP-stimulated exocytic delivery of NKCC2. We silenced VAMP2 expression *in vivo* with shRNAs and measured exocytic delivery of

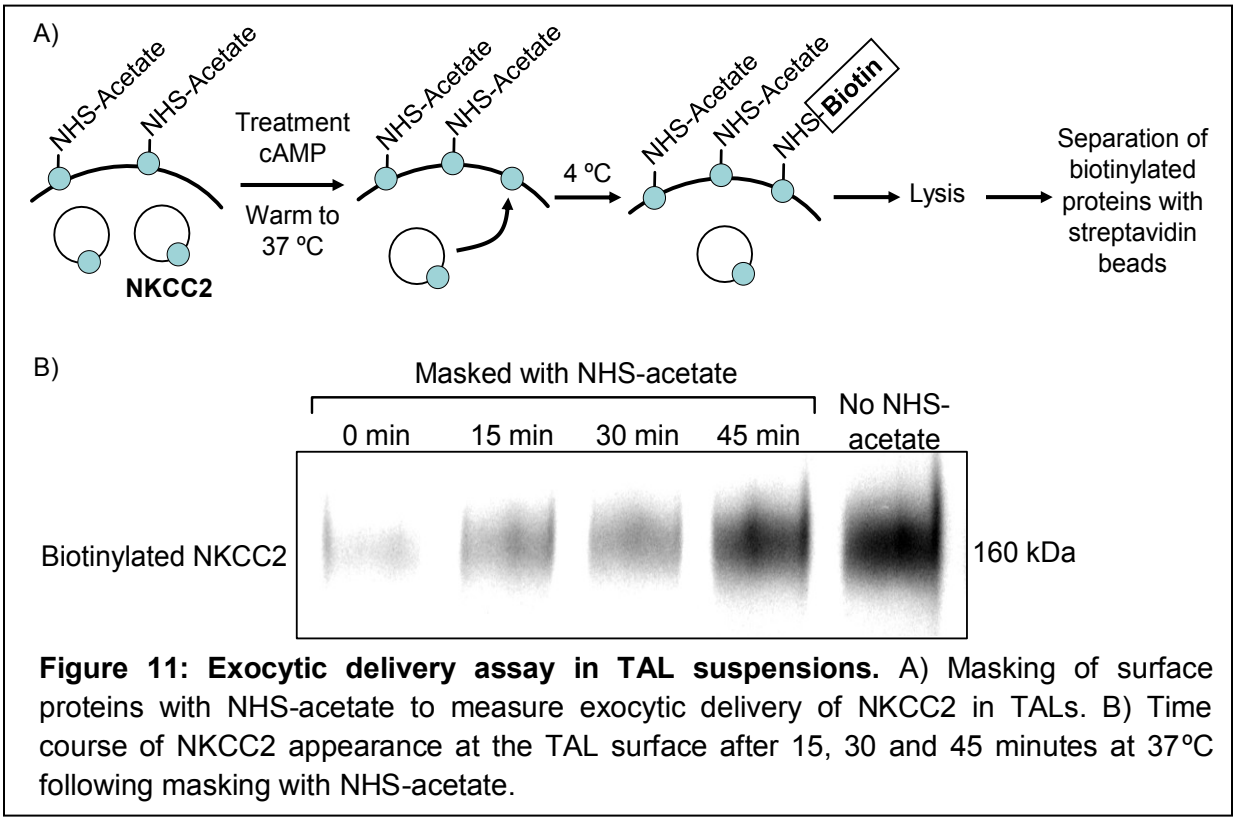

Figure 11: Exocytic delivery assay in TAL suspensions. A) Masking of surface proteins with NHS-acetate to measure exocytic delivery of NKCC2 in TALs. B) Time course of NKCC2 appearance at the TAL surface after 15, 30 and 45 minutes at 37ºC following masking with NHS-acetate.

NKCC2 over 30 minutes in TALs by a modified surface biotinylation procedure. This procedure involves a previous step of masking surface biotinylation sites with an NHS-acetate reagent. The NHS group reacts with extracellular lysines in surface proteins, blocking the sites accessible to NHS-SS-biotin (Figure 11a). NHS-acetate does not cross the cell membrane, so it does not bind intracellular NKCC2. When TALs are warmed at 37ºC, intracellular NKCC2 is delivered to the cell surface and accessible for biotinylation. We next purified the biotinylated fraction, which represents newly delivered NKCC2. The representative Western blot in Figure 11b shows the signal for surface NKCC2 with and without NHS-acetate blockade (first and last lanes respectively). The difference between these two lanes is the NHS-acetate blocked fraction and was used as a reference to measure exocytic delivery. Figure 11b shows the gradual appearance of surface Figure 11b shows NKCC2 after 15, 30 and 45 minutes at 37ºC.

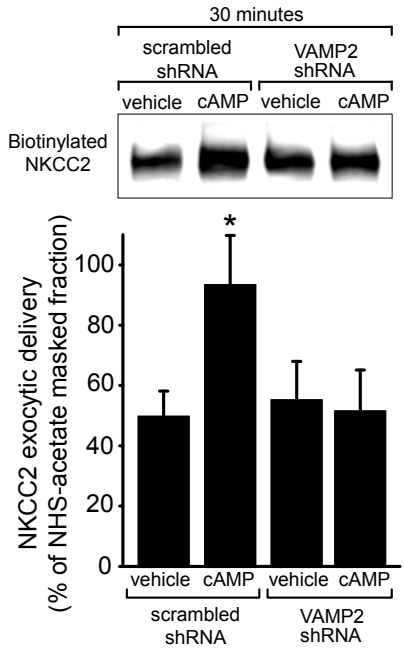

Figure 12: VAMP2 silencing blocked cAMP-stimulated but not constitutive exocytic delivery of NKCC2 in TALs. *p<0.05 *vs.* vehicle, scrambled-shRNA.

In scrambled shRNA-transduced TALs, baseline NKCC2 exocytic delivery over 30 minutes averaged 49 ± 9% of the NHS-acetate-masked fraction (Figure 12). When cAMP was stimulated with forskolin + IBMX, NKCC2 exocytic delivery rose to 93 ± 16% of the NHS-acetate-masked fraction, a 90% increase (p<0.05; n =5). In TALs transduced with VAMP2-shRNA, cAMP-stimulated NKCC2 exocytic delivery was completely blocked, whereas baseline NKCC2 delivery was not affected (vehicle: 55 ± 13% *vs.* cAMP: 51 ± 14%, p<0.05). As

expected, intracellular control GAPDH was not detected in the surface fraction (not shown). These data indicate that VAMP2 mediates all of the cAMP-stimulated NKCC2 exocytic delivery without affecting constitutive delivery of NKCC2 to the cell surface.

VAMP2 silencing does not decrease total NKCC2 expression.

To assure that the decrease in steady-state surface NKCC2 expression we observed is not due to decreased NKCC2 expression, we silenced VAMP2 *in vivo* in TALs and measured total NKCC2 expression by Western blot. We did not observe a significant change in total NKCC2 expression after silencing VAMP2 in TALs (scrambled-shRNA: 100% *vs.* VAMP2-shRNA: 86 ± 7%; n = 7) (Figure 13), indicating that VAMP2 is not required for normal NKCC2 expression.

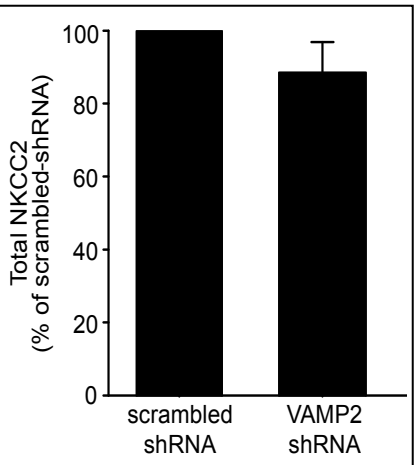

Figure 13: Silencing VAMP2 did not decrease total NKCC2 expression in TALs.

VAMP2 silencing does not decrease NKCC2 phosphorylation at Serine 126

Our data indicate that VAMP2 mediates cAMP-stimulated NKCC2 exocytic delivery. Previous data from another group indicate that cAMP phosphorylates NKCC2 at Ser-126 *via* PKA (72). We have shown that PKA mediates cAMP-stimulated NKCC2 exocytic delivery (77). To test the possibility that VAMP2 silencing interferes with cAMP-stimulated PKA signaling to NKCC2, we measured whether VAMP2 silencing decreases total NKCC2 phosphorylation at Ser-126. We measured NKCC2 phosphorylation with a phospho-Ser-126 specific antibody (72). We used the cAMP analogue db-cAMP (250 μM) to better control cAMP levels. As expected, baseline NKCC2 phosphorylation was almost undetectable, but it increased by 69 ± 14 fold after treatment with db-cAMP (p<0.01; n = 4)

in control scrambled-shRNA-transfected TALs (Figure 14). We observed that after silencing VAMP2, db-cAMP strongly enhanced Ser-126 phosphorylation by 73 ± 24 fold (p<0.01 *vs.* vehicle). This stimulation was not significantly different between control and VAMP2-shRNA-transduced TALs. These data indicate that VAMP2 silencing does not decrease the ability of cAMP to stimulate PKA and phosphorylate NKCC2.

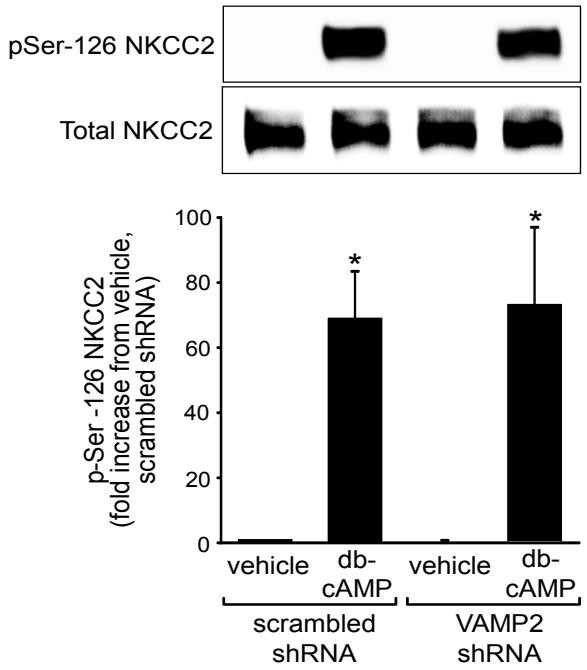

Figure 14: Silencing VAMP2 did not decrease cAMP-stimulated NKCC2 phosphorylation at Ser-126. *p<0.01 *vs.* vehicle.

Conclusion

We observed that in addition to VAMP2 and VAMP3, also VAMP7 and VAMP8 are expressed in the TAL. In this dissertation we focused in VAMP2 and VAMP3 since they are the targets of tetanus toxin. In this aim we studied VAMP2 because it mediates cAMP-stimulated exocytosis in other cells. We concluded that VAMP2 mediates cAMP-stimulated steady-state surface NKCC2 expression and cAMP-stimulated exocytic delivery of NKCC2 in medullary TALs. VAMP2 does not mediate constitutive steady-state surface NKCC2 expression, constitutive NKCC2 exocytic delivery or is required for total NKCC2 expression. We also concluded that the ability of cAMP to stimulate PKA and phosphorylate NKCC2 at Serine-126 is not affected by VAMP2 silencing. The significance and in depth interpretation of all these observations will be addressed in the discussion in Chapter 4.

Aim 2- Hypothesis: cAMP stimulates NKCC2-VAMP2 interaction and co-localization in thick ascending limbs.

Rationale

In aim 1 we showed that VAMP2 mediates cAMP-stimulated NKCC2 trafficking. However, the mechanism by which this occurs remains unexplored. One possible mechanism may involve protein-protein interactions, since NKCC2 interacts with proteins that regulate its trafficking to the plasma membrane (163,165,166). Also, VAMP2 physically interacts with potassium (159,160) and calcium channels (158) in neurons. These interactions modulate channel activity. In this aim we will test whether NKCC2 interacts with VAMP2 and we will discuss the relevance of this interaction for NKCC2 function.

We have previously shown that cAMP stimulation enhances apical membrane exocytosis in TALs (77). If VAMP2 is in the same vesicles as NKCC2, it is expected that cAMP will also promote delivery of VAMP2 to the apical surface. In this aim we also quantified the rate at which cAMP stimulates delivery of VAMP2 to the TAL surface. We also studied whether once at the surface, VAMP2 displays a localization pattern that overlaps with NKCC2's.

In neurons, phosphorylation is a mechanism to regulate SNARE interaction with calcium channels and this regulates neurotransmitter release (156,176). However, none of the kinases described to date are cAMP-dependent. The focus of this aim is to study how cAMP affects NKCC2-VAMP2 interaction as a possible component of the mechanism that mediates VAMP2 control over cAMP-stimulated NKCC2 trafficking.

Results

Co-immunoprecipitation of NKCC2 and VAMP2 in thick ascending limbs

Previous data from our laboratory indicated that NKCC2 co-localizes with VAMP2 in an intracellular sub-apical location in TALs (14). To test whether NKCC2 interacts with VAMP2, we performed co-immunoprecipitation assays in fresh TAL lysates obtained from rat renal outer medullas. We observed that VAMP2 co-immunoprecipitated with NKCC2 in TALs whereas VAMP7 and VAMP8 did not (Figure 15a, n = 4). To confirm these results, we performed the reciprocal immunoprecipitation with an anti-VAMP2 antibody and detected NKCC2 by Western blot (Figure 15b, n= 4).

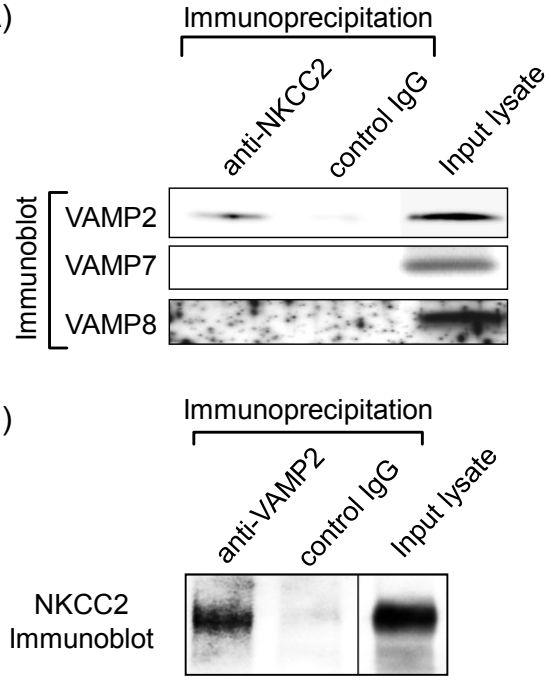

Figure 15: NKCC2 and VAMP2 co-immunoprecipitate in TALs. A) VAMP2 but not VAMP7 and VAMP8 co-immunoprecipitated with NKCC2. B) Reciprocal immunoprecipitation with anti-NKCC2 antibody confirmed interaction with VAMP2 in TAL lysates.

Controls with non-immune IgG showed absence of NKCC2, VAMP2, VAMP7 or VAMP8. These data indicate that VAMP2 is a protein binding partner of NKCC2 in TALs. However, it is not known whether this interaction is direct or *via* intermediate proteins.

Interaction of VAMP2 with the carboxy-terminus of NKCC2

To further define the region of NKCC2 that interacts with VAMP2, we designed the two GST-fusion proteins shown in Figure 16a. We fused GST to the amino-terminus of NKCC2 (GST-N-NKCC2) or a 71 amino acid-long sequence in the carboxy-terminus (GST-C-NKCC2). This carboxy-terminal region has previously been shown to contain an apical

localization signal (177). It was chosen for our study because the proteins MAL (163) and moesin (164) bind this region and mediate NKCC2 trafficking to the cell surface. We expressed the GST-fusion proteins in bacteria (Figure 16b) and used them as bait to pull down VAMP2 from TAL lysates. We observed that VAMP2 interacted with GST-C-NKCC2 but not with GST-N-NKCC2 or GST alone (Figure 16c, n = 3), indicating that VAMP2 interacts with NKCC2 at the carboxy-terminal region that contains the signal for apical localization.

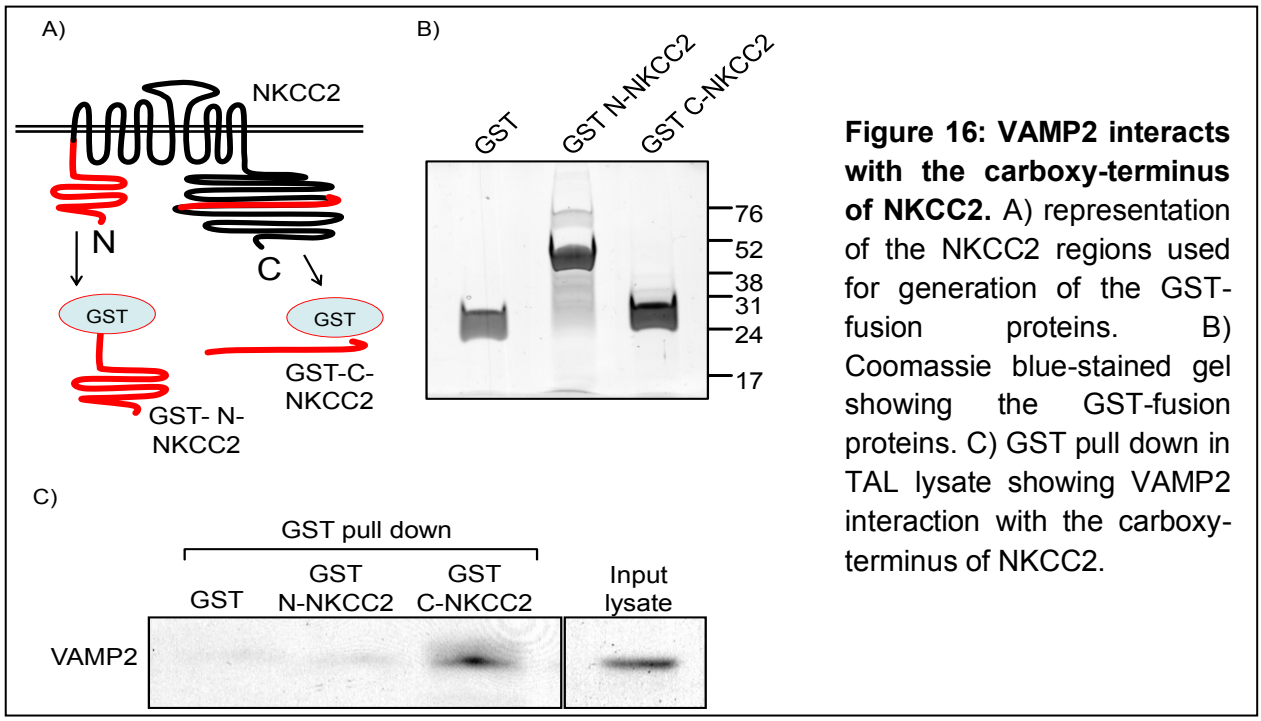

Figure 16: VAMP2 interacts with the carboxy-terminus of NKCC2. A) representation of the NKCC2 regions used for generation of the GST-fusion proteins. B) Coomassie blue-stained gel showing the GST-fusion proteins. C) GST pull down in TAL lysate showing VAMP2 interaction with the carboxy-terminus of NKCC2.

Generation and characterization of thick ascending limb cell primary culture from rat renal medulla

To study the localization of VAMP2 and NKCC2 at the apical surface of TALs, we developed a primary culture procedure for TAL cells. We obtained TAL cells from rat renal outer medullas and cultured them in permeable support coated with a basement membrane extract to promote cell polarization (Figure 17a). After three days, cells achieved 100% confluence and expressed NKCC2 (Figure 17b). Since NKCC2 is a TAL marker, this

indicates the culture is differentiated.

We further characterized the TAL culture by assessing whether NKCC2 is directed to the apical membrane. This cell culture setup allowed us to have direct access to the apical surface. We performed apical surface biotinylation and we found that NKCC2 was expressed at the apical surface in the same proportion as in native TALs. In addition, cAMP stimulation for 30 minutes increased steady-state surface NKCC2 expression similar to native TALs without changing intracellular NKCC2 expression (Figure 17b, n = 3). Intracellular protein GAPDH was not detected in the surface fraction.

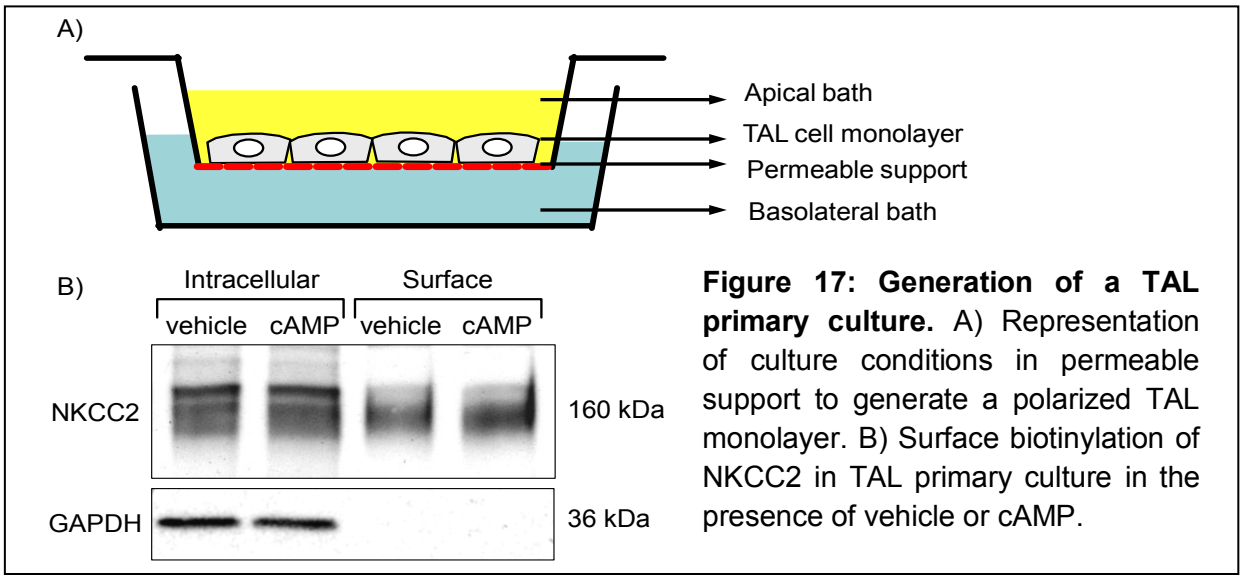

Figure 17: Generation of a TAL primary culture. A) Representation of culture conditions in permeable support to generate a polarized TAL monolayer. B) Surface biotinylation of NKCC2 in TAL primary culture in the presence of vehicle or cAMP.

We also observed a prominent band of a slightly higher molecular weight (≈180 kDa) in the intracellular fraction. It is not uncommon to observe this higher band in fresh TAL suspensions and it may correspond to an immature glycosylated form of NKCC2. It is known that NKCC2 is heavily glycosylated in the extracellular loop between transmembrane domains 7 and 8, producing a shift in molecular weight from the predicted 120 kDa to the 160 kDa corresponding to mature NKCC2 (178). The immature 120 kDa band is almost undetectable in Western blots (173). The ≈180 kDa band may correspond to incomplete trimming or the sugar residues during NKCC2 maturation, although this has

never been addressed. In native TAL suspensions, the ≈180 kDa is not as strong as the one we observed in TAL cultures compared to the 160 kDa band. This may indicate incomplete maturation of NKCC2 in primary cultures. However, the ≈180 kDa band almost disappeared in the surface fraction, suggesting that only mature NKCC2 reaches the surface, making the TAL primary cultures a suitable model to study NKCC2 trafficking.

To study the localization of NKCC2 at the apical surface, we labeled surface NKCC2 with an exofacial antibody that recognizes an extracellular epitope in NKCC2 (24). As represented in Figure 18a, since the cells are not permeabilized, the antibody does not label intracellular NKCC2. We next inverted the culture and mounted it in order to image the cells at different focal planes by confocal microscopy as shown in Figure 18b. We observed expression of NKCC2 at a region about 1 µm deep from the apical surface (Figure 18c). Interestingly, surface NKCC2 was distributed in a heterogeneous pattern

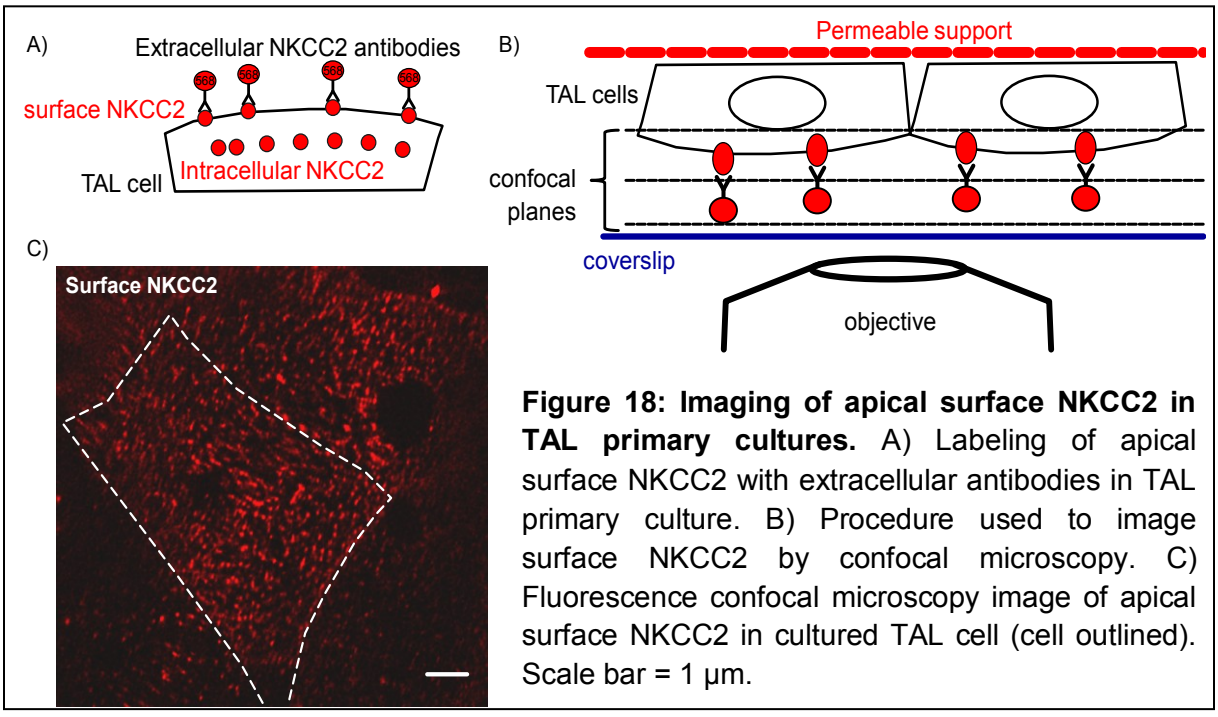

Figure 18: Imaging of apical surface NKCC2 in TAL primary cultures. A) Labeling of apical surface NKCC2 with extracellular antibodies in TAL primary culture. B) Procedure used to image surface NKCC2 by confocal microscopy. C) Fluorescence confocal microscopy image of apical surface NKCC2 in cultured TAL cell (cell outlined). Scale bar = 1 µm.

restricted to clusters or discrete domains at the apical surface. To our knowledge, this pattern of distribution of NKCC2 at the TAL apical surface has never been observed before.

Altogether, these data indicate that in TAL primary cultures NKCC2 retains polarity, the mature form is directed to the apical membrane and responds to cAMP in the same way as native TALs.

NKCC2 and VAMP2 co-localization at the apical surface of thick ascending limbs

Because we found that NKCC2 and VAMP2 interact in TALs, we next tested whether they co-localize at the apical surface. Once a vesicle fuses, VAMP2 remains at the plasma membrane for some time before being internalized, since it contains a transmembrane domain. This makes possible to detect VAMP2 at the apical surface of TAL primary cultures. However, the portion of VAMP2 that faces the extracellular space after exocytosis is only a few amino acids long and is not possible to label with antibodies. To label VAMP2 at the apical surface we followed the procedure represented in Figure 19.

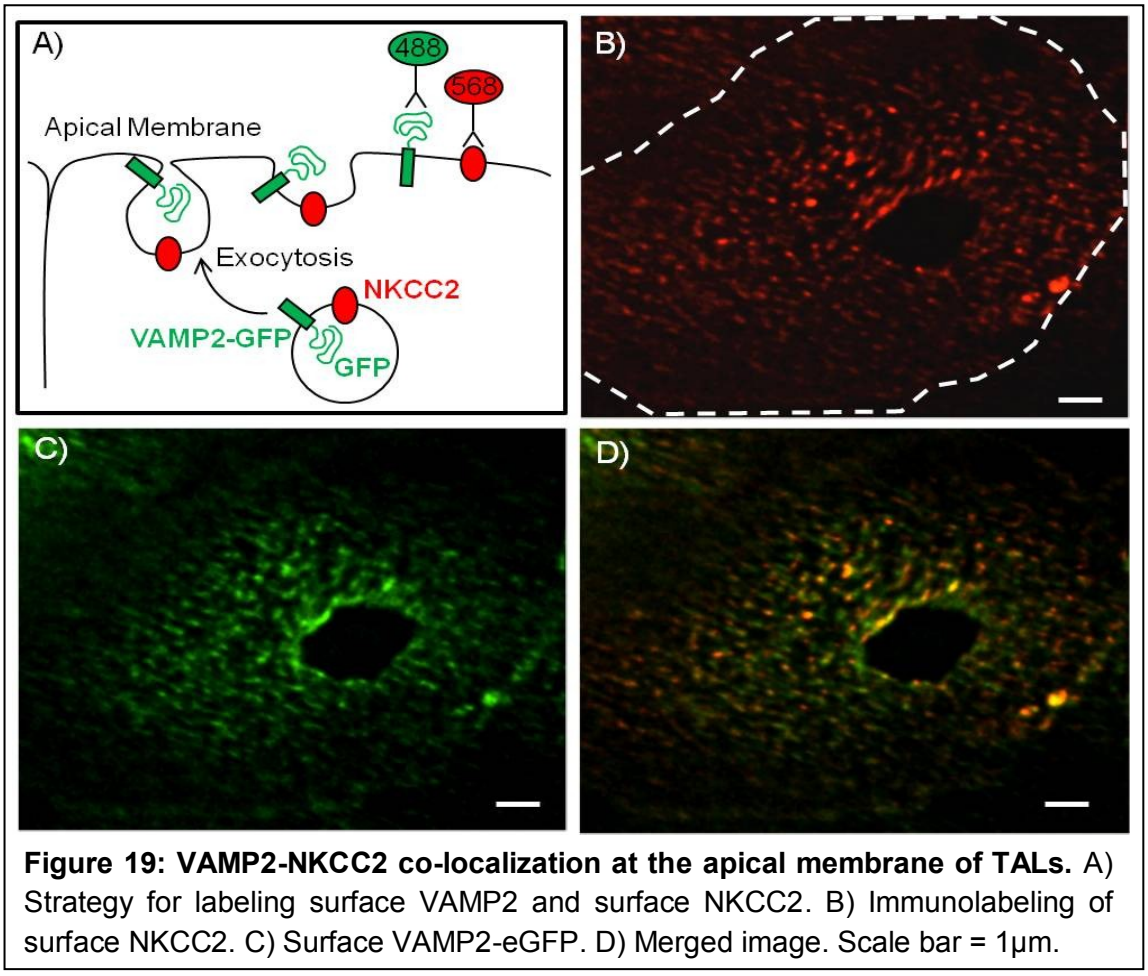

Figure 19: VAMP2-NKCC2 co-localization at the apical membrane of TALs. A) Strategy for labeling surface VAMP2 and surface NKCC2. B) Immunolabeling of surface NKCC2. C) Surface VAMP2-eGFP. D) Merged image. Scale bar = 1µm.

We transfected TAL cells in culture with a VAMP2 construct fused to eGFP in the carboxy-terminus (179). When vesicles carrying VAMP2-eGFP undergo exocytosis, the GFP tag faces the extracellular space (Figure 19a). Then, cells were cooled to 4°C and incubated with an extracellular anti-GFP antibody (green) and the exofacial anti-NKCC2 antibody (red), to label surface VAMP2 and surface NKCC2 respectively. The antibodies do not label intracellular proteins since cells are not permeabilized. Imaging conditions were adjusted so intrinsic fluorescence from GFP was not detectable. We observed that surface NKCC2 (Figure 19b) and surface VAMP2 (Figure 19c) localized at the apical cell surface in a heterogeneous pattern forming discrete clusters. After image deconvolution, we different determined the degree of co-localization by measuring the Mander's overlap coefficient. Figure 20 shows images of the same cell where co-localizing pixels were obtained at stringency conditions. A high Mander's overlap coefficient (closer to 1) is represented in Figure 20a and is the most stringent condition. However, this criterion would overlook co-localization between pixels of different intensity, which can vary with the labeling and image acquisition conditions. On the other hand, a low Mander's overlap coefficient (0.7) (Figure 20b) would relax the stringency of the measurement and overestimate the co-localization. We used a Mander's overlap coefficient equal or higher than 0.95 to measure

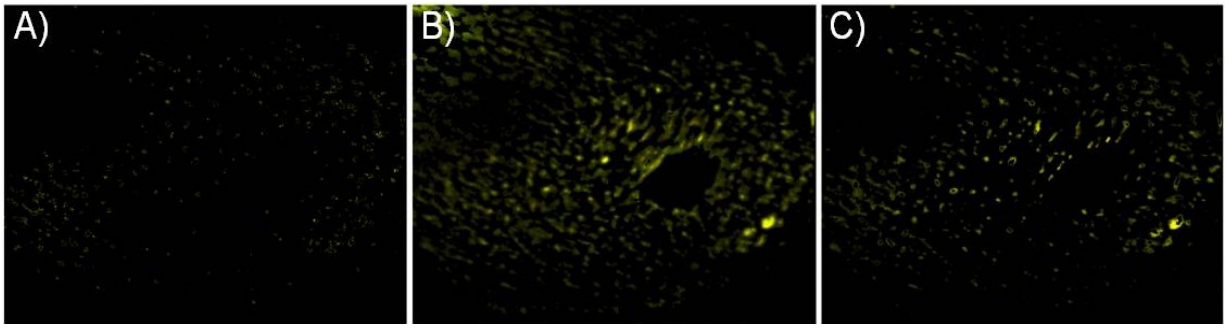

Figure 20: Comparison of different co-localization stringency criteria. Mander's overlap coefficients of 0.99 (A), 0.7 (B) and 0.95 (C) were applied to the same picture to generate the different images of co-localizing pixels.

co-localization at the apical surface (Figure 20c). We counted the number of NKCC2 and co-localizing clusters and determined that 47 ± 8% of NKCC2 clusters at the apical surface also contained VAMP2 (n = 5). It is important to notice that since labeling is performed in a single plane at the cell surface, artifacts due to signal originated at different planes do not occur in our setup. These data indicate that VAMP2 and NKCC2 are located in similar clusters or micro domains at the apical surface of TAL cells.

Effect of cAMP on NKCC2-VAMP2 co-localization at the apical surface of thick ascending limbs

We described in the previous aim that cAMP-stimulated NKCC2 exocytic delivery is mediated by VAMP2. We also observed that NKCC2 and VAMP2 interact and co-localize at the apical surface of TAL. We next tested whether NKCC2-VAMP2 co-localization at the apical surface is enhanced by cAMP. We transfected TAL primary cultures with VAMP2-eGFP and then treated them with vehicle or forskolin + IBMX for 20 minutes to enhance cAMP. Next we stained NKCC2 and VAMP2-eGFP at the apical surface and quantified the number of apical clusters in which NKCC2 and VAMP2 co-localized (Figure 21). We

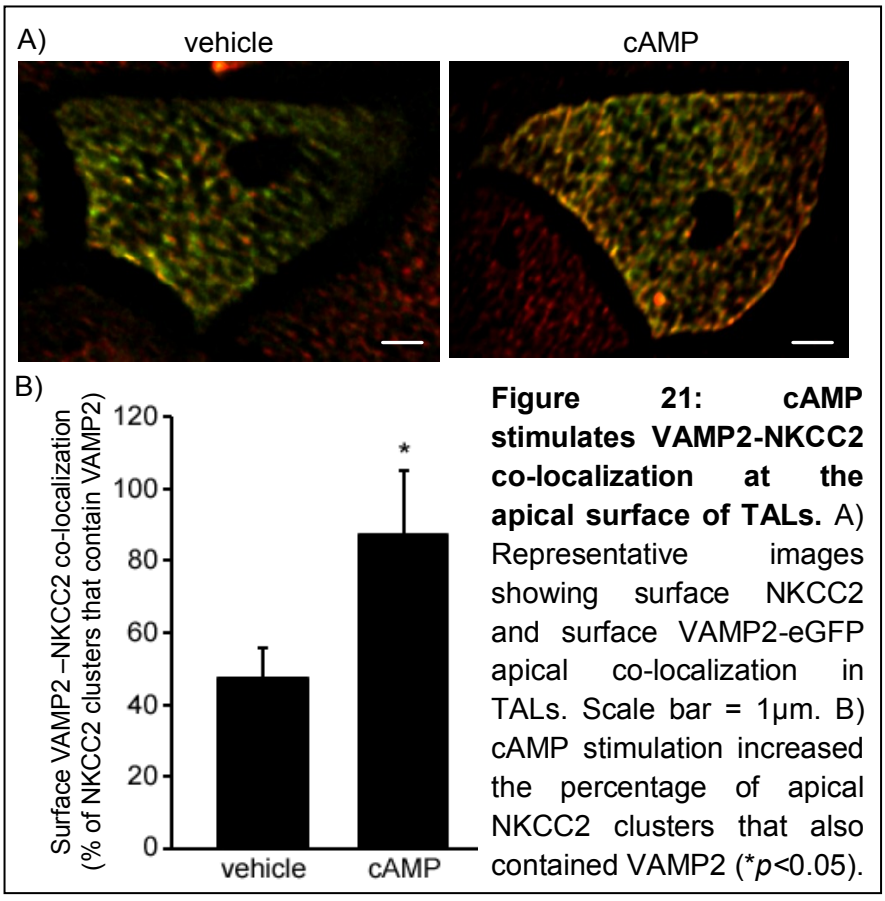

Figure 21: cAMP stimulates VAMP2-NKCC2 co-localization at the apical surface of TALs. A) Representative images showing surface NKCC2 and surface VAMP2-eGFP apical co-localization in TALs. Scale bar = 1μm. B) cAMP stimulation increased the percentage of apical NKCC2 clusters that also contained VAMP2 (*p<0.05).

observed that cAMP stimulation increased the number of NKCC2 clusters that also contained VAMP2 from 47 ± 8% to 87 ± 18% (p<0.05, n = 8), indicating that cAMP increased NKCC2-VAMP2 co-localization at the apical surface of TALs.

Effect of cAMP on VAMP2 exocytic delivery in thick ascending limbs

We found that VAMP2 is at the apical membrane of TALs and mediates cAMP-stimulated NKCC2 exocytic delivery. Since cAMP stimulates NKCC2-VAMP2 co-localization at the membrane, it is possible that cAMP also stimulates delivery of VAMP2 to the apical surface. To test this possibility, we transfected TAL primary cultures with the VAMP2-eGFP construct and measured the time course of VAMP2-eGFP exocytic delivery at 20 and 30 minutes by surface biotinylation (Figure 22a). Masking surface proteins with NHS-acetate decreased biotinylated VAMP2-eGP signal by 78 ± 1%, similar to what we observed previously for NKCC2 (77). Stimulation of cAMP with forskolin + IBMX increased the rate of VAMP2-eGFP exocytic delivery by more than 80% (vehicle: 4.8 ± 0.9 arbitrary units per minute *vs*. cAMP: 8.7 ± 1.4 arbitrary units per

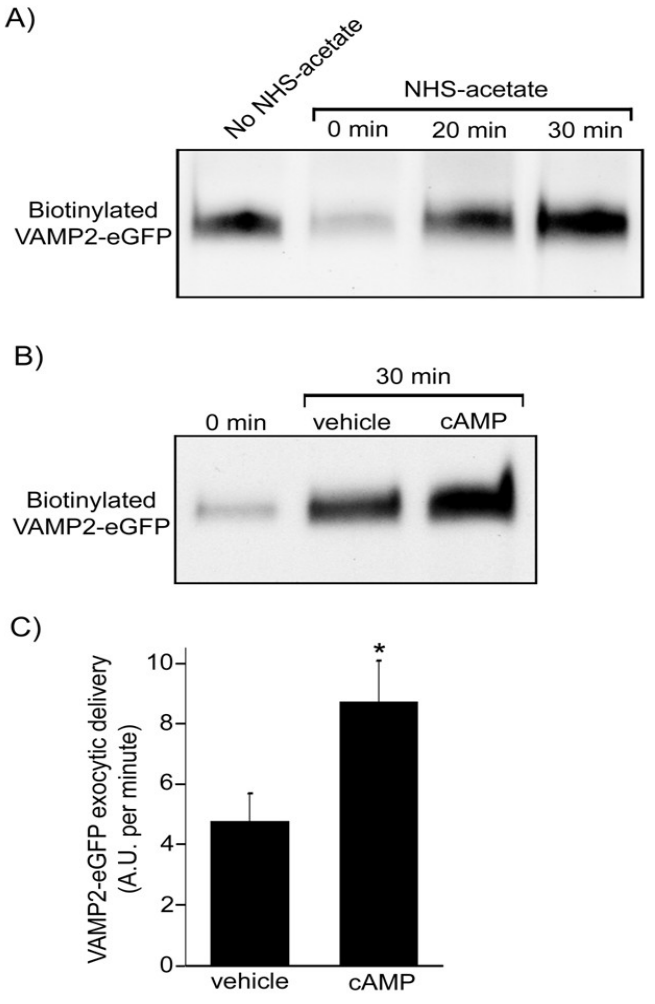

Figure 22: cAMP stimulates VAMP2 exocytic delivery in TALs. A) Time course of VAMP2-eGFP exocytic delivery. B) Representative blot showing increase in VAMP2-eGFP exocytic delivery in the presence of cAMP. C) cAMP increased VAMP2-eGFP exocytic delivery in TALs.

minute, $p<0.05$, n = 4) (Figures 22b and 22c). These results indicate that cAMP stimulates VAMP2 exocytic delivery in TALs.

Effect of cAMP on VAMP2-NKCC2 interaction

VAMP2 silencing did not affect NKCC2 phosphorylation by PKA, but it blocked exocytic delivery. Previously we found that PKA mediates cAMP-stimulated NKCC2 exocytic delivery, suggesting that targets of PKA other than NKCC2 might influence the interaction of NKCC2 with VAMP2. Thus we tested whether stimulation of PKA enhanced this interaction. For this, we co-immunoprecipitated NKCC2 and VAMP2 from TALs treated with vehicle or the PKA agonist N^6-Benzoyl-cAMP + IBMX for 30 minutes. We found that PKA stimulation increased the amount of VAMP2 pulled down by NKCC2 immunoprecipitation (Figure 23). No interaction with non-immune IgG was detected, and no change in either total VAMP2 or NKCC2 expression was observed during treatment with the PKA agonist. These data indicate that PKA stimulation enhanced NKCC2-VAMP2 interaction in TALs.

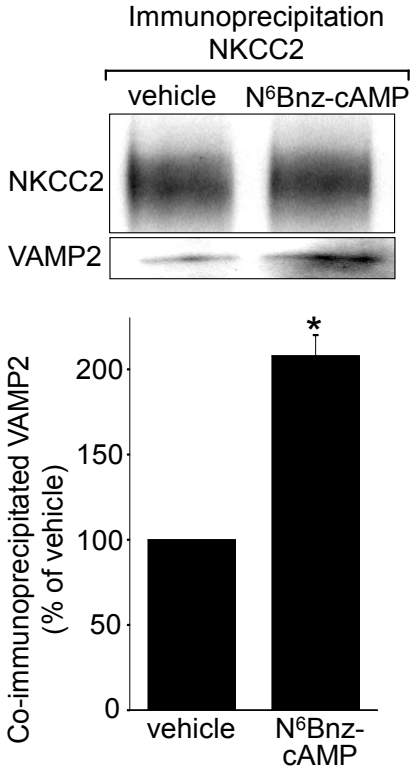

Figure 23: PKA stimulation enhances VAMP2-NKCC2 interaction in TALs. (*$p<0.01$).

Conclusion

We concluded that NKCC2 and VAMP2 interact in TALs and PKA stimulation by cAMP enhances this interaction as shown by co-immunoprecipitation. We identified the region of NKCC2 that interacts with VAMP2 as a carboxy-terminal segment previously known to mediate apical targeting of NKCC2. To study NKCC2 co-localization with VAMP2 at the apical surface of TALs, we developed primary cultures that retain characteristics of

differentiated TALs. We labeled NKCC2 and VAMP2 at the apical surface and measured co-localization. We observed that NKCC2 and VAMP2 co-localize at the cell surface in apical clusters or micro domains. This allowed us to determine that NKCC2 and VAMP2 remain at a similar localization after they are exocytosed. Whether they physically interact at the apical surface is not clear from the co-localization analysis. The co-immunoprecipitation and the GST pull down experiments indicate that the two proteins physically interact, either directly or *via* intermediate proteins. However the cellular compartment where this interaction occurs was not possible to determine in our study.

Stimulation with cAMP enhanced VAMP2-NKCC2 co-localization at apical surface clusters. We also observed that cAMP stimulated VAMP2 delivery to the apical TAL surface. Together with enhanced NKCC2-VAMP2 interaction in the presence of cAMP, our data prompt us to speculate that protein-protein interactions may be part of the mechanism by which VAMP2 mediates cAMP-stimulated NKCC2 trafficking.

CHAPTER 3

ROLE OF VAMP3 IN CONSTITUTIVE NKCC2 TRAFFICKING IN THE THICK ASCENDING LIMB AND RENAL FUNCTION

Introduction

We have previously shown that NKCC2 undergoes constitutive exocytic delivery in the absence of any stimulation (77). Since VAMP2 inhibition did not affect baseline steady-state surface NKCC2 and exocytic delivery, some other VAMP isoform must mediate constitutive trafficking of NKCC2. Another observation from the previous chapter is that, in addition to VAMP2, the TAL also expresses three other VAMP isoforms. Two of them, VAMP7 and VAMP8, known as endobrevins, are usually involved in intracellular vesicle-vesicle fusion events (145,146), and are unlikely to mediate delivery of NKCC2 to the plasma membrane. The fourth isoform expressed in the TAL is VAMP3, which mediates exocytic events in most cells studied to date (150,180-185). In this chapter we addressed the role of VAMP3 in constitutive NKCC2 trafficking. We divided this chapter into two additional aims. In aim 3 we tested whether VAMP3 mediates constitutive trafficking of NKCC2 in the TAL. In aim 4, following the approach we used for VAMP2, we studied whether VAMP3 and NKCC2 interact. In addition, we addressed the role of VAMP3 in mediating normal renal function and blood pressure.

Aim 3- Hypothesis: VAMP3 mediates constitutive trafficking of NKCC2 in the thick ascending limb.

Rationale

Tetanus toxin exclusively cleaves VAMP2 and VAMP3, since these are the only two proteins that contain the cleavage site for the toxin. If VAMP3 mediates constitutive

NKCC2 trafficking, we should observe a decrease in surface NKCC2 after treatment with tetanus toxin. Our observations from aim 1 indicate that VAMP2 does not mediate constitutive trafficking of NKCC2. Therefore, any effect of tetanus toxin on baseline steady-state surface NKCC2 can be attributed to VAMP3. In this aim we studied whether tetanus toxin decreases constitutive steady-state surface NKCC2 expression. We tested the hypothesis that VAMP3 mediates constitutive trafficking of NKCC2 in the thick ascending limb. To specifically address the role of VAMP3, we used silencing shRNAs and a VAMP3 knockout mouse model to study NKCC2 trafficking.

Results

Effect of tetanus toxin on constitutive steady-state surface NKCC2 in thick ascending limbs

We treated TAL suspensions with 30 nM tetanus toxin for 50 minutes and measured steady-state surface NKCC2 expression by surface biotinylation in the absence of stimulation (Figure 24a). Intracellular GAPDH was not biotinylated. We observed that

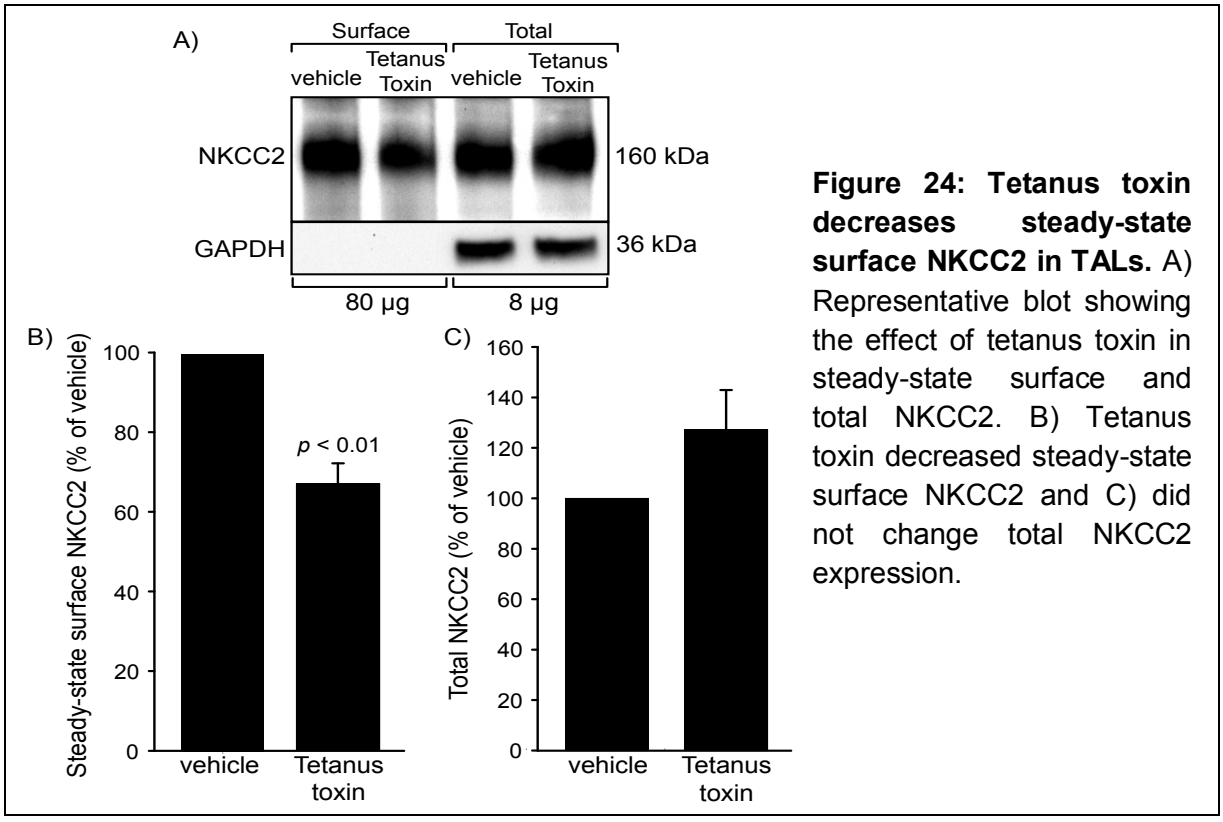

Figure 24: Tetanus toxin decreases steady-state surface NKCC2 in TALs. A) Representative blot showing the effect of tetanus toxin in steady-state surface and total NKCC2. B) Tetanus toxin decreased steady-state surface NKCC2 and C) did not change total NKCC2 expression.

constitutive steady-state surface NKCC2 was decreased by 33 ± 5% compared to vehicle (Figure 24b, n = 6). Tetanus toxin did not decrease total NKCC2 expression (Figure 24c). These data indicate that constitutive NKCC2 trafficking to the TAL surface is mediated by a tetanus toxin-sensitive VAMP. In order to address whether this VAMP isoform is VAMP3, we silenced VAMP3 expression in TALs.

VAMP3 silencing *in vitro* and *in vivo*

To directly address the role of VAMP3 in NKCC2 trafficking, we designed silencing RNAs. We followed the same protocol we developed in aim 1 for VAMP2. We observed

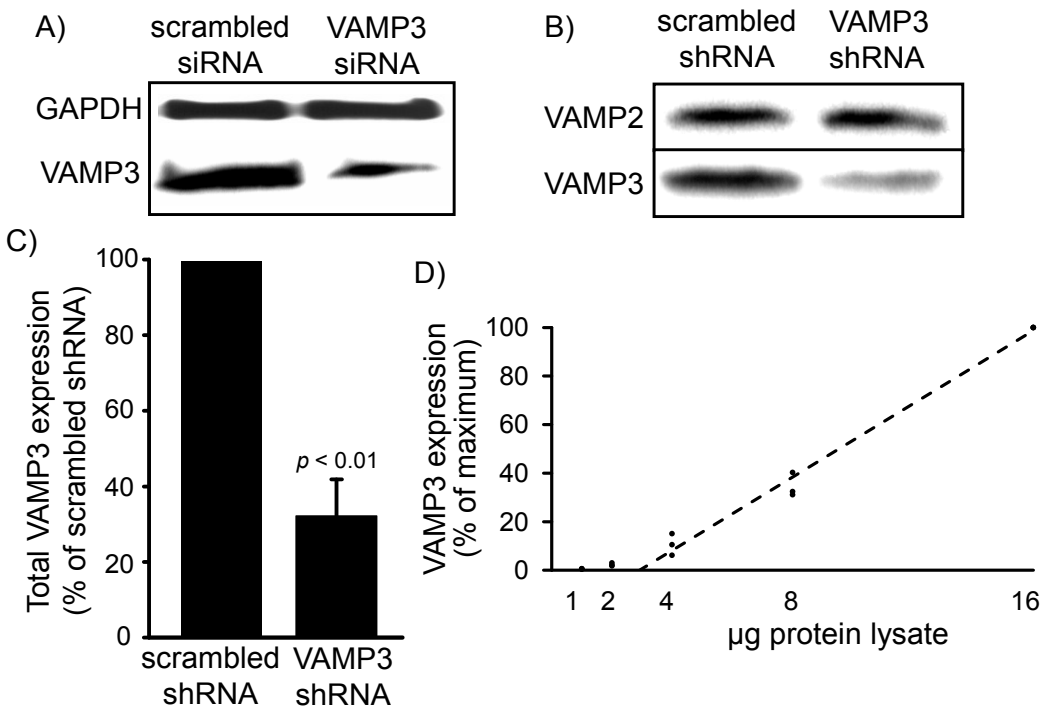

Figure 25: VAMP3 silencing *in vitro* and *in vivo.* A) VAMP3 silencing *via* siRNA in NRK-52E cells. B) VAMP2 and VAMP3 expression after silencing VAMP3 in TALs. C) Decrease in VAMP3 expression after silencing in TALs. D) Curve of VAMP3 protein expression in TALs ($R^2 = 0.995$).

efficient and specific silencing of VAMP3 in NRK-52E cells (Figure 25a) and in medullary TAL suspensions (Figure 25b). We did not observe any decrease in VAMP2 expression. After 72 hours of silencing, we achieved a decrease of 68 ± 10% in VAMP3 protein

expression compared with the contra lateral kidney injected with control scrambled-shRNA (p<0.01, n = 3) (Figure 25c). The curve depicted in Figure 25d shows the linear range used in our protein measurements. Altogether these data indicate that VAMP3-shRNAs were efficient and specific for VAMP3 silencing.

Effect of VAMP3 silencing on NKCC2 exocytic delivery

We next tested whether silencing VAMP3 inhibits constitutive exocytic delivery of NKCC2 in TALs. In TALs transduced with scrambled-shRNA, baseline NKCC2 exocytic delivery over 30 minutes averaged 49 ± 9% of the NHS-acetate-masked fraction, and it was enhanced to 92 ± 13% with cAMP stimulation (p<0.05, n = 5) (Figure 26). When we silenced VAMP3, constitutive exocytic delivery at 30 minutes was almost completely blocked (7 ± 6% of the NHS-acetate-masked fraction). However cAMP was still able to stimulate NKCC2 delivery (61 ± 16% of the NHS-acetate-masked fraction).

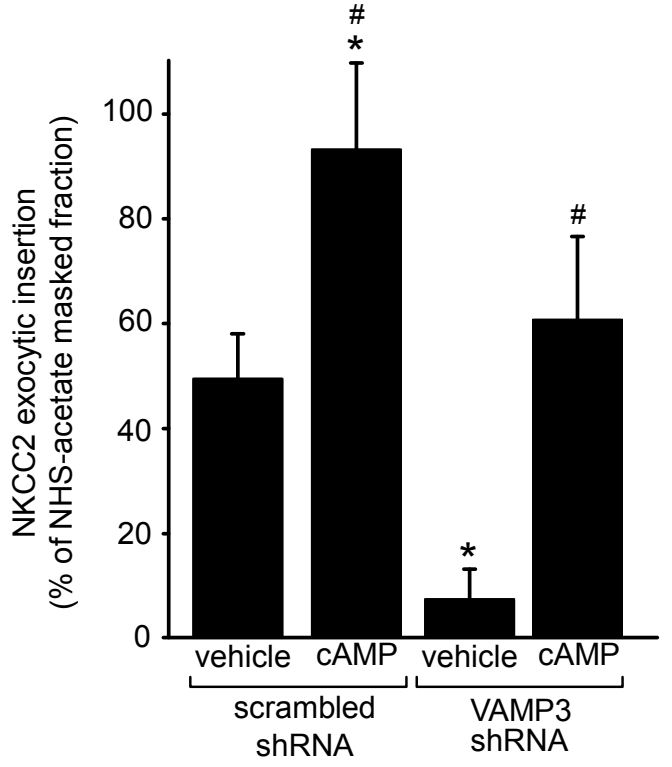

Figure 26: Silencing VAMP3 in TALs blocked constitutive but not cAMP-stimulated exocytic delivery of NKCC2. (*p<0.05 *vs.* vehicle, scrambled shRNA; #p<0.05 *vs.* vehicle).

There may be a confounding factor in our measurements arising from the lower constitutive exocytic delivery of NKCC2 when we silenced VAMP3. Since the measurements for cAMP-stimulated exocytic delivery are generated by comparing to a lower baseline number, this could lead to overestimation of the stimulation with cAMP

under VAMP3 silencing. Indeed, from our numbers, cAMP stimulated exocytic delivery of NKCC2 by 0.9 fold in control TALs and by 7.7 fold in VAMP3-silenced TALs. This could be misinterpreted as VAMP3 inhibiting cAMP-stimulated NKCC2 delivery. To circumvent this, we can consider the absolute values to obtain a measurement in arbitrary units and subtract the difference between vehicle- and cAMP-treated TALs. Then we divide this difference by the time transcurred (30 minutes) to obtain a rate of exocytic delivery. By doing this we observed that the exocytic delivery rate in control TALs was 1.4 ± 0.1 arbitrary units per minute and in VAMP3-silenced TALs it was of 1.8 ± 0.3 arbitrary units per minute. A difference that was not statistically significant.

Overall, these data indicate that VAMP3 mediates constitutive but not cAMP-stimulated NKCC2 delivery in TALs.

Effect of VAMP3 silencing on steady-state surface NKCC2

If silencing VAMP3 blocks NKCC2 exocytic delivery, this should be reflected in decreased steady-state surface NKCC2 expression. To test this, we silenced VAMP3 in

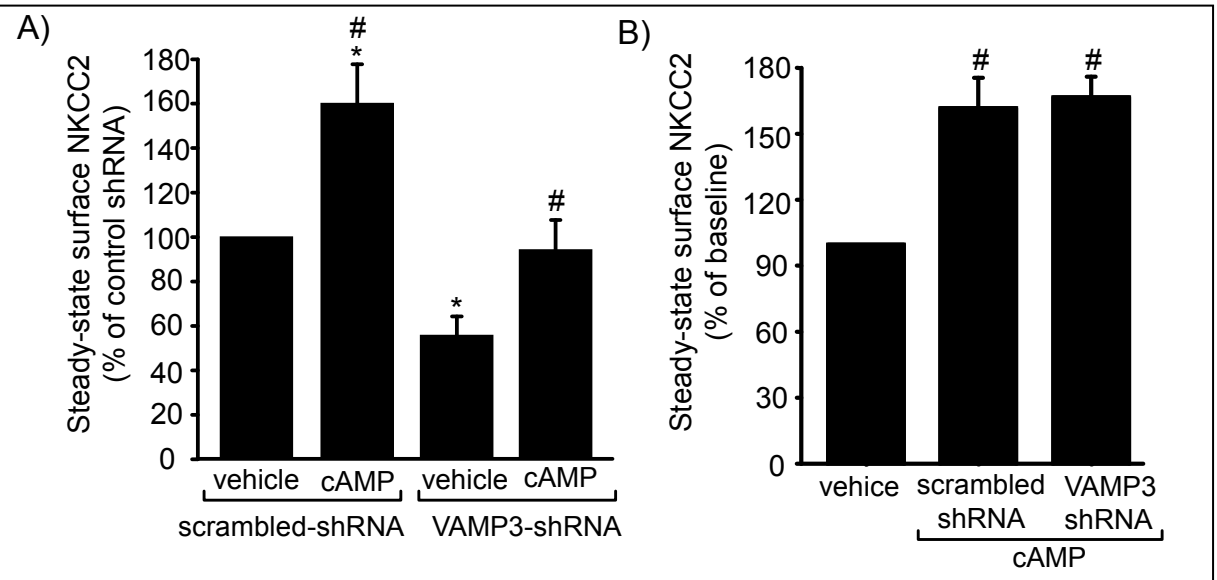

Figure 27: Silencing VAMP3 decreased constitutive but not cAMP-stimulated steady-state surface NKCC2 expression in TALs. A) Steady-state surface NKCC2 in scrambled-shRNA and VAMP2-shRNA transduced TALs. B) cAMP-stimulated steady-state surface NKCC2 (*p<0.05 *vs.* scrambled-shRNA, vehicle; # p<0.05 *vs.* vehicle).

TALs and measured steady-state surface NKCC2 by surface biotinylation. We observed a 43% decrease in constitutive steady-state surface NKCC2 (scrambled-shRNA: 100% *vs.* VAMP3-shRNA: 57 ± 7%, $p<0.05$, n = 7) (Figure 27a). In contrast, cAMP-stimulated steady-state surface NKCC2 was not affected. We quantified the magnitude of this response and observed that cAMP stimulated steady-state surface NKCC2 to the same extent in TALs transfected with scrambled-shRNA (62 ± 14% stimulation) and with VAMP3-shRNA (67 ± 9% stimulation) (Figure 27b). These data indicate that VAMP3 mediates constitutive but not cAMP-stimulated steady-state surface NKCC2 expression, which is primarily mediated by VAMP2.

Effect of VAMP3 silencing on total NKCC2 expression.

We showed that silencing VAMP3 prevents NKCC2 delivery to the plasma membrane in TALs. However, silencing VAMP3 can also affect NKCC2 expression. To address this possibility, we silenced VAMP3 and measured total NKCC2 protein in TALs. Interestingly, silencing VAMP3 decreased total NKCC2 expression by 45% (scrambled-shRNA: 100% *vs*. VAMP3-shRNA: 55 ± 6 %, $p<0.01$, n = 7) (Figure 28). This observation suggests that VAMP3 is required to maintain normal levels of NKCC2 protein in TALs.

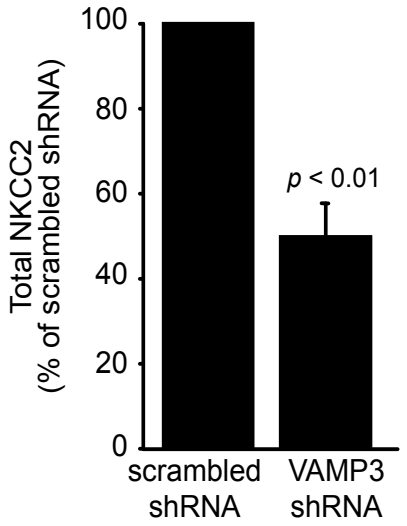

Figure 28: Silencing VAMP3 decreased total NKCC2 expression in TALs.

NKCC2 expression and steady-state surface levels in VAMP3 knockout mice.

Since VAMP3 mediates NKCC2 trafficking and is also required for normal NKCC2 expression, we would expect that in VAMP3 knockout mice total NKCC2 and steady- state surface expression are decreased. We obtained medullary TAL suspensions from VAMP3

knockout mice and measured: a) VAMP3 protein to assure that it is not expressed, b) total NKCC2 expression and c) steady-state surface NKCC2 expression.

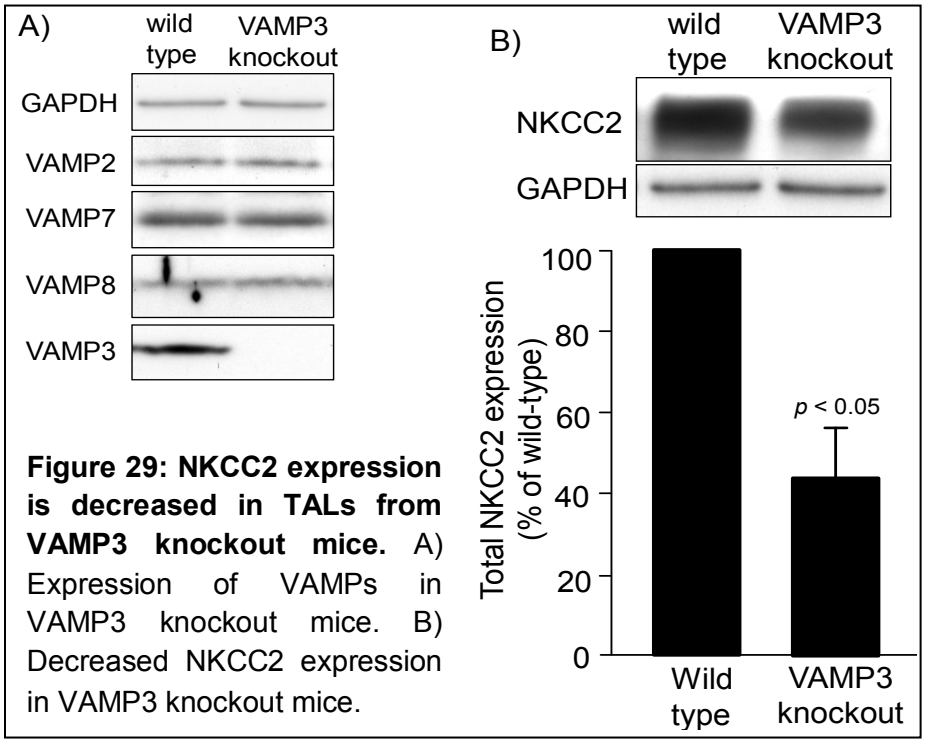

Figure 29: NKCC2 expression is decreased in TALs from VAMP3 knockout mice. A) Expression of VAMPs in VAMP3 knockout mice. B) Decreased NKCC2 expression in VAMP3 knockout mice.

We did not detect any VAMP3 expression in TALs from VAMP3 knockout mice, and there was no change in expression of VAMP2, VAMP7 or VAMP8 (Figure 29a, n = 3). Total NKCC2 expression was decreased by 66 ± 12% (p<0.05, n = 6) compared to wild-type mice (Figure 29b) and steady-state surface NKCC2 was decreased by 46% (wild-type = 100% *vs.* VAMP3 knockout = 54 ± 4, p<0.05, n = 3) (Figure 30), consistent with the VAMP3 silencing results. Altogether these observations confirm that VAMP3 mediates steady-state surface NKCC2 expression and normal total NKCC2 expression.

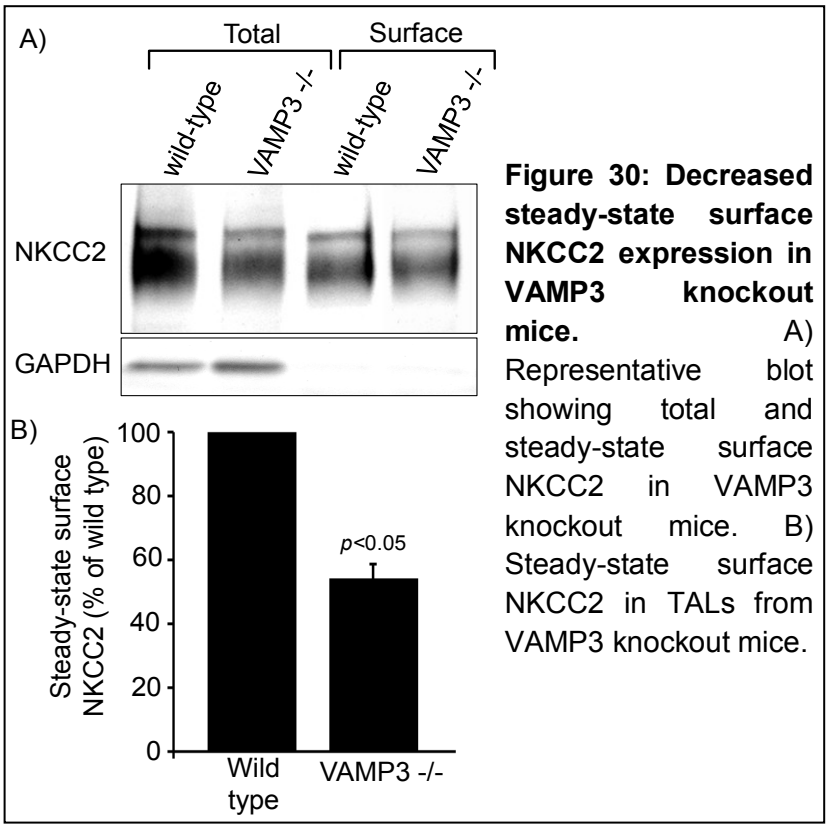

Figure 30: Decreased steady-state surface NKCC2 expression in VAMP3 knockout mice. A) Representative blot showing total and steady-state surface NKCC2 in VAMP3 knockout mice. B) Steady-state surface NKCC2 in TALs from VAMP3 knockout mice.

Conclusion

We concluded that VAMP3 mediates constitutive NKCC2 exocytic delivery and steady-state surface NKCC2 expression. Stimulation by cAMP was not affected by VAMP3 silencing in TALs. Interestingly, silencing VAMP3 also decreased total NKCC2 protein, indicating that VAMP3 is required for normal NKCC2 expression. The mechanism by which VAMP3 mediates normal NKCC2 expression was not directly addressed in this dissertation. However, it may involve maturation of intracellular compartments or re-routing of NKCC2 vesicles that cannot reach the plasma membrane when VAMP3 is silenced. These possibilities will be discussed in detail in Chapter 4.

The previous observations during VAMP3 silencing were confirmed in TALs from VAMP3 knockout mice, where total NKCC2 and baseline steady-state surface NKCC2 expression were also decreased. This role of VAMP3 explains why tetanus toxin decreases constitutive steady-state surface NKCC2 expression in TALs. Altogether, out results indicate that the constitutive pathway for NKCC2 trafficking is controlled independently from the cAMP-stimulated pathway and is mediated by VAMP3.

Aim 4- Hypothesis: VAMP3 interacts with NKCC2 in the thick ascending limb and maintains normal renal function and blood pressure.

Rationale

In the previous aim we showed that VAMP3 mediates constitutive NKCC2 trafficking and is required for normal NKCC2 expression. We also showed in aim 2 that VAMP2 interacts with NKCC2. In this aim we tested whether VAMP3 also interacts with NKCC2 and whether they co-localize at the apical surface of TALs.

Since NKCC2 plays a crucial role in renal physiology, we would expect that renal

function is altered in VAMP3 knockout mice. Other investigators have not been able to discern any major phenotype in VAMP3 knockout mice (186-188). However, to our knowledge, renal function has never been evaluated in these mice. To correlate the alterations we observed in NKCC2 with decreased renal function and blood pressure, we measured urine excretion and systolic arterial pressure in VAMP3 knockout mice.

Results

Co-immunoprecipitation of NKCC2 and VAMP3 in thick ascending limbs

To determine whether NKCC2 interacts with VAMP3 in TALs, we immunoprecipitated NKCC2 from TAL lysates and detected VAMP3 by Western blot. We observed that VAMP3 co-immunoprecipitated with NKCC2 (Figure 31a, n = 4). A control non-immune IgG failed to precipitate VAMP3. Next, we performed the reciprocal experiment by immunoprecipitating VAMP3 and detecting NKCC2 (Figure 31b, n = 4). The interaction was confirmed as revealed by NKCC2 being present in the samples incubated with anti-VAMP3 IgG but not with non-immune IgG. These data indicate that VAMP3 interacts with NKCC2 in TALs.

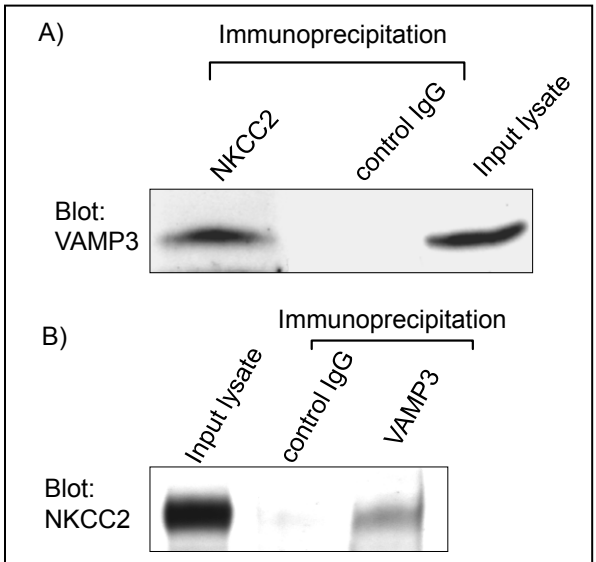

Figure 31: VAMP3 co-immunoprecipitates with NKCC2 in TALs. A) VAMP3 co-immunoprecipitated with NKCC2. B) Reciprocal immunoprecipitation with anti-NKCC2 antibody.

NKCC2 and VAMP3 co-localization at the apical surface of thick ascending limbs

Since VAMP3 interacts with NKCC2, we next tested whether they co-localize at the apical membrane of TALs. We transfected TAL cultures with adenoviruses carrying

VAMP3-eGFP and measured surface expression of VAMP3-eGFP and NKCC2 by fluorescence confocal microscopy as described in aim 2 for VAMP2. We observed that VAMP3 was also restricted to discrete domains at the apical membrane (Figure 32). We next examined the areas that exhibited co-localization. We observed that VAMP3 and NKCC2 co-localized in 19 ± 4% of the apical clusters that contain NKCC2 (p<0.05, n = 5). These data indicate that VAMP3 and NKCC2 share localization at micro domains in the apical surface of TAL cells, although the degree of co-localization was less than what we observed with VAMP2.

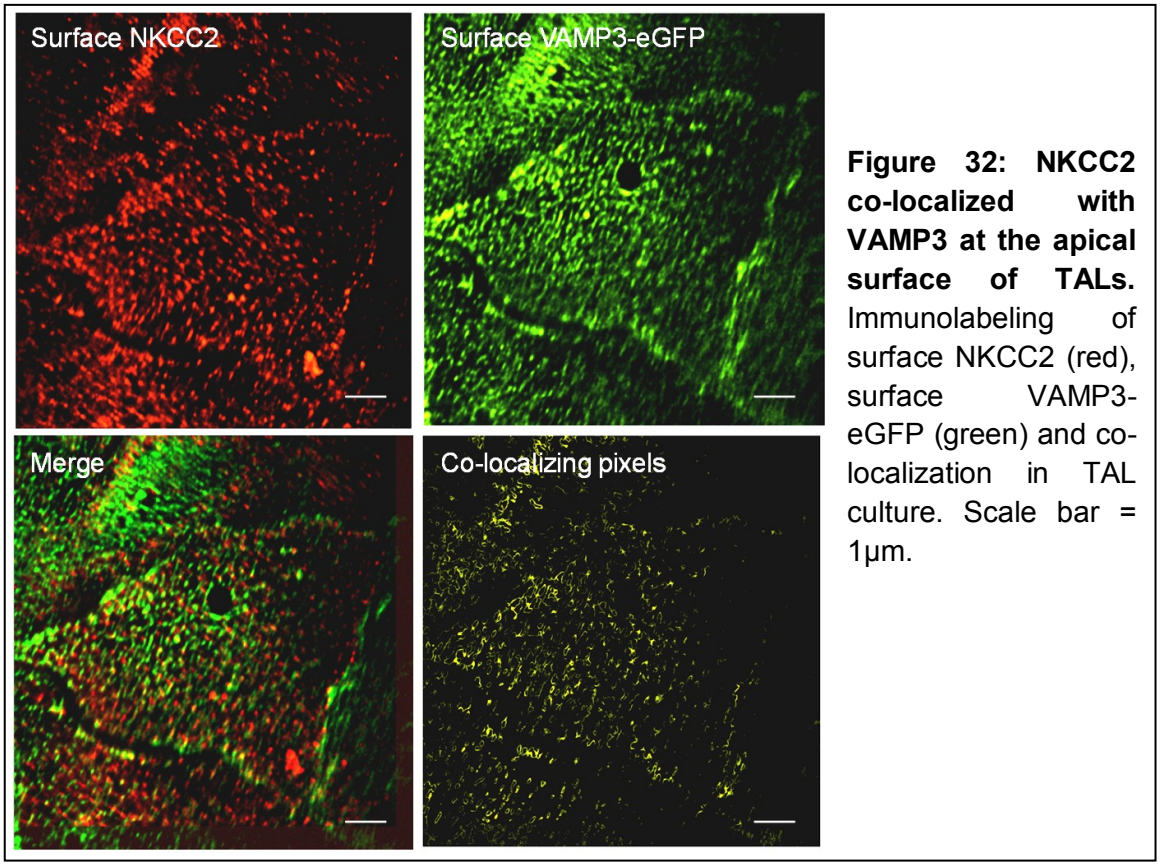

Figure 32: NKCC2 co-localized with VAMP3 at the apical surface of TALs. Immunolabeling of surface NKCC2 (red), surface VAMP3-eGFP (green) and co-localization in TAL culture. Scale bar = 1μm.

Baseline urinary excretion in VAMP3 knockout mice

We showed that VAMP3 mediates NKCC2 trafficking in TALs and also that VAMP3 knockout mice have decreased NKCC2 expression and impaired steady-state surface NKCC2 expression. To correlate these observations with a renal phenotype, we measured

urine volume, osmolality and electrolyte excretion in VAMP3 knockout mice. The results are summarized in Table 1. We observed that compared to wild-type, VAMP3 knockout mice excreted a slightly higher urine volume, produced more diluted urine and excreted more Ca (p<0.05, n = 6), consistent with a TAL defect. We did not observe any difference in urinary excretion of Na, Cl and K. Although this resembles the phenotype of NKCC2 knockout mice (40), we would expect enhanced excretion of these ions if TAL function was impaired. To test how VAMP3 mice adjust urinary excretion to disturbances of water and electrolyte homeostasis, we challenged them to water deprivation or low-salt diet in the next experiments.

Table 1: Urine parameters in wild-type and VAMP3 knockout mice.

	wild-type	VAMP3 knockout
water consumed (ml/24 hs)	4.7 ± 0.2	5.1 ± 0.3
urine volume (ul/24 hs)	1337 ± 118	1625 ± 167
urine osmolality (mOsm)	3433 ± 166	2924 ± 131 *
UNa (µmol/day)	264.8 ± 18.9	266.3 ± 31.2
UK (µmol/day)	779.8 ± 51.2	781.9 ± 98.2
UCl (µmol/day)	541.4 ± 43.7	611.5 ± 65.8
UCa (µmol/day)	0.60 ± 0.02	0.81 ± 0.05 *
UMg (µmol/day)	1.9 ± 0.19	1.73 ± 0.13
UCreat. (mg/day)	0.31 ± 0.02	0.35 ± 0.04

** p<0.05*

Urinary excretion in VAMP3 knockout mice during 24 hour water deprivation

To further test how renal function in VAMP3 knockout mice responds to a water restriction challenge, we deprived them of water for 24 hours. In wild type mice, urine volume decreased from 1.34 ± 0.12 ml to 0.65 ± 0.08 ml (p<0.05) as expected. Urine volume also decreased in VAMP3 knockout mice from 1.62 ± 0.17 ml to 0.67 ± 0.08 ml (p<0.05), indicating that VAMP3 knockouts can still prevent fluid loss in the urine when water supply is limited. Urine osmolality increased in wild type mice from 3433 ± 166

mOsm to 5307 ± 235 mOsm (p<0.05). This response is expected since decreased excretion of water would produce more concentrated urine. VAMP3 knockout mice, besides initially producing a more diluted urine of 2924 ± 131 mOsm, they could still concentrate the urine (p<0.05) to an osmolality comparable with wild types under water deprivation (5763 ± 456 mOsm). This suggests that VAMP3 do not experience more water loss than wild-types when they are deprived of drinking water for 24 hours.

Since we did not observe any major change in urinary Na, Cl and K under baseline conditions, we next tested whether excretion of these ions changed after water deprivation. In wild type and in knockout mice, urinary Na, Cl and K were increased following water deprivation (Figure 33). This is a normal response independent of vasopressin (189,190), probably mediated by atrial natriuretic peptide released from the heart in response to oxytocin (191-193). We will expand this in the general discussion in Chapter 4. In VAMP3 knockout mice, urinary Na, Cl and K were also increased during water deprivation but to a greater extent than in wild-type mice (p<0.05, n = 6), indicating that VAMP3 knockouts excrete excess Na, Cl and K when deprived of water.

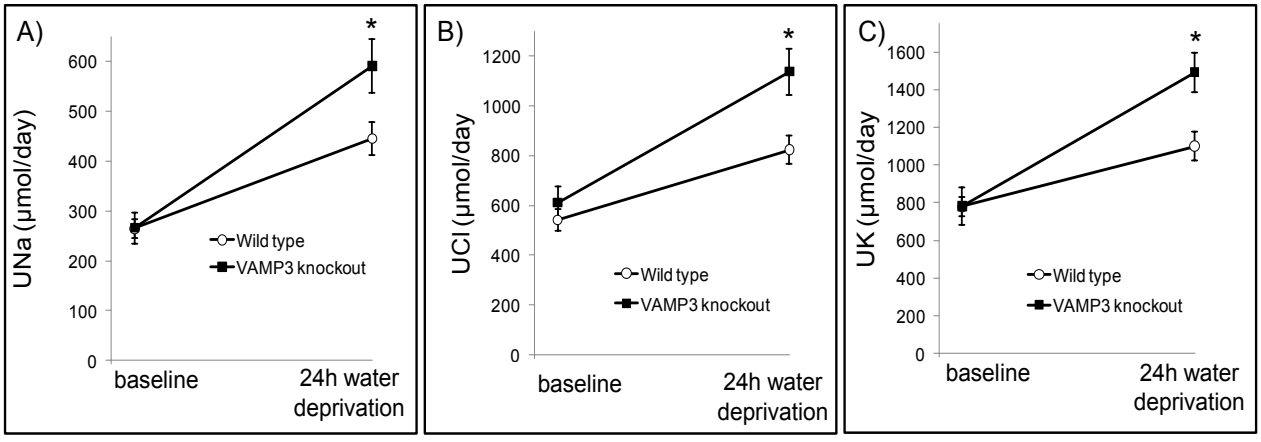

Figure 33: Enhanced urinary Na, Cl and K excretion in VAMP3 knockout mice during water restriction. A) 24-hour urinary Na excretion, B) 24-hour urinary Cl excretion, C) 24-hour urinary K excretion during 24-hour water deprivation (*p<0.05 *vs.* wild-type).

Altogether these data indicate that VAMP3 mediates normal excretion of Na, Cl and K under water deprivation, but it is not required for concentrating the urine under the same conditions.

Urinary Na excretion in VAMP3 knockout mice under low-sodium diet

When Na intake is restricted, it is expected that urinary Na excretion would be decreased since renal function would adjust to retain the limited amount of available Na. To test whether VAMP3 knockout can properly adjust Na excretion when Na supply is limited, we fed them a low-Na diet (0.02% Na) for 24 and 48 hours. We observed that urinary Na excretion was decreased to 39 ± 8 µmol/day after 24 hours and to 15 ± 6 µmol/day after 48 hours on low Na diet (p<0.05, n = 6) (Figure 34). In VAMP3 knockout mice, urinary Na excretion was also decreased after 24 hs on low-salt diet (59 ± 12 µmol/day) but this was significantly higher than wild type mice. After 48 hours on low-Na diet, urinary Na excretion was 20 ± 8 µmol/day, not statistically different from wild type mice. These data indicate that VAMP3 knockout mice take longer to adjust urinary excretion of Na when Na supply in the diet is limited.

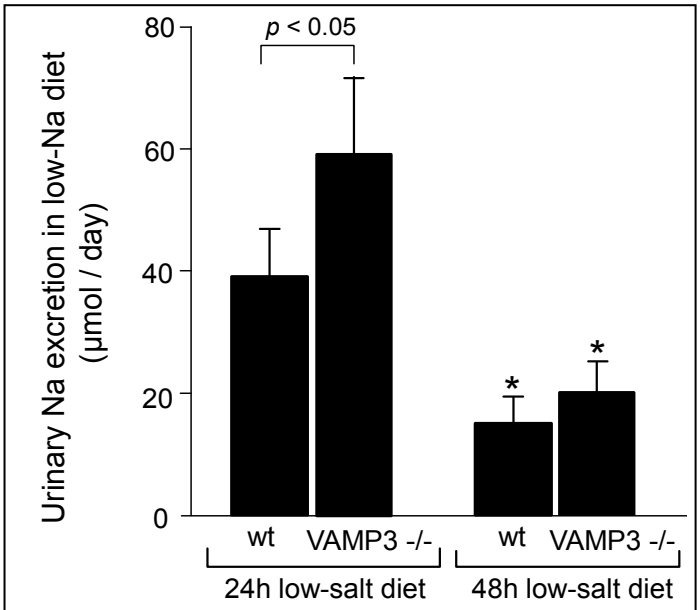

Figure 34: Delayed adjustment of urinary Na excretion in VAMP3 knockout mice on a low-sodium diet. (*p<0.05 *vs.* 24 hour low-salt diet).

Blood pressure in VAMP3 knockout mice

If VAMP3 is required for NKCC2 function and for normal fluid and electrolyte reabsorption, VAMP3 knockout mice may exhibit lower blood pressure. To correlate the

renal phenotype we observed with differences in blood pressure, we measured systolic arterial pressure in VAMP3 knockout mice by tail cuff. We observed that wild-type mice had an arterial pressure of 114 ± 2 mmHg, while arterial pressure in VAMP3 knockouts was 19 mmHg lower (95 ± 2 mmHg, $p<0.05$, n = 6) (Figure 35). These results indicate that VAMP3 is required to maintain normal blood pressure in mice.

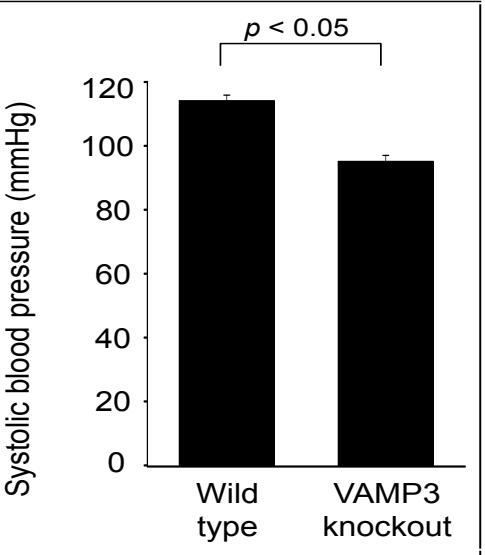

Figure 35: Decreased systolic blood pressure in VAMP3 knockout mice.

Conclusion

We concluded that VAMP3 interacts with NKCC2 as shown by co-immunoprecipitation in TAL protein lysates. Whether NKCC2-VAMP3 interaction is part of the mechanism by which VAMP3 mediates NKCC2 trafficking is not clear, but it is a possibility as discussed for VAMP2. Also, whether NKCC2 and VAMP3 interact at the surface or at an intracellular localization was not tested. Although we also observed VAMP3 co-localization with NKCC2 at apical clusters in the TAL surface, this only indicates close proximity but not necessarily direct physical interaction. However, our data indicate that NKCC2 and VAMP3 are directed to the same membrane localization, in agreement with the role of VAMP3 on NKCC2 trafficking we described in aim 3.

We showed for the first time that genetic deletion of VAMP3 causes a renal phenotype. VAMP3 knockout mice excrete slightly higher urine volume, produce less concentrated urine and excrete excess Ca under baseline conditions. These data are consistent with decreased TAL reabsorptive capacity and with the phenotype observed in NKCC2 knockout mice (40). In addition, when VAMP3 knockout mice were challenged to

water deprivation conditions, they failed to retain Na, Cl and K compared to wild-type mice in spite of still being able to concentrate the urine. The Na loss in the urine was also evident after 24 hours in low Na diet, but it recovered to normal levels after 48 hs, indicating that renal Na excretion took longer to adjust. Finally, VAMP3 knockout mice had lower systolic blood pressure as expected from the excess fluid and electrolyte excretion we observed in the urine. Altogether, we conclude that VAMP3 is required for normal renal function and maintenance of blood pressure. The phenotype of VAMP3 knockout mice will be discussed in detail and in a broader context later in Chapter 4.

CHAPTER 4

CONCLUDING REMARKS

Summary of results

In this study we have shown that:

1. VAMP2, VAMP3, VAMP7 and VAMP8 are expressed in the TAL.
2. VAMP2 mediates cAMP-stimulated exocytic delivery of NKCC2 to the TAL surface while VAMP3 mediates constitutive delivery of NKCC2 to the apical surface.
3. VAMP2 mediates cAMP-stimulated steady-state surface NKCC2 expression in TALs while VAMP3 mediates constitutive steady-state surface NKCC2 expression.
4. Tetanus toxin decreases constitutive steady-state surface NKCC2 expression.
5. VAMP2 and VAMP3 co-immunoprecipitate with NKCC2 in TALs.
6. VAMP2 interacts with a region of the carboxy-terminus of NKCC2 previously shown to determine apical targeting.
7. NKCC2 is distributed in discrete micro domains at the apical surface of TALs.
8. VAMP2 and VAMP3 co-localize with NKCC2 at micro domains in the apical surface of TALs.
9. cAMP stimulates VAMP2 exocytic delivery in TALs, enhances VAMP2 presence at the apical surface, and promotes NKCC2-VAMP2 interaction.
10. VAMP3 is required for normal NKCC2 expression, urine concentrating ability, water and salt reabsorption, and to maintain normal blood pressure.

Proposed model

Based on our data we propose the model depicted in Figure 36. In TALs, NKCC2 may be segregated into two intracellular pools. One pool is associated with VAMP2 and responds to cAMP stimulation to be delivered into the apical membrane. The other pool

is associated with VAMP3 and undergoes constitutive delivery. When VAMP3 is blocked, vesicles carrying NKCC2 cannot reach the apical membrane and they may be re-routed for degradation. The consequences of interfering with the components of this process are: decreased renal absorption of water and ions and abnormal blood pressure, as observed in VAMP3 knockout mice.

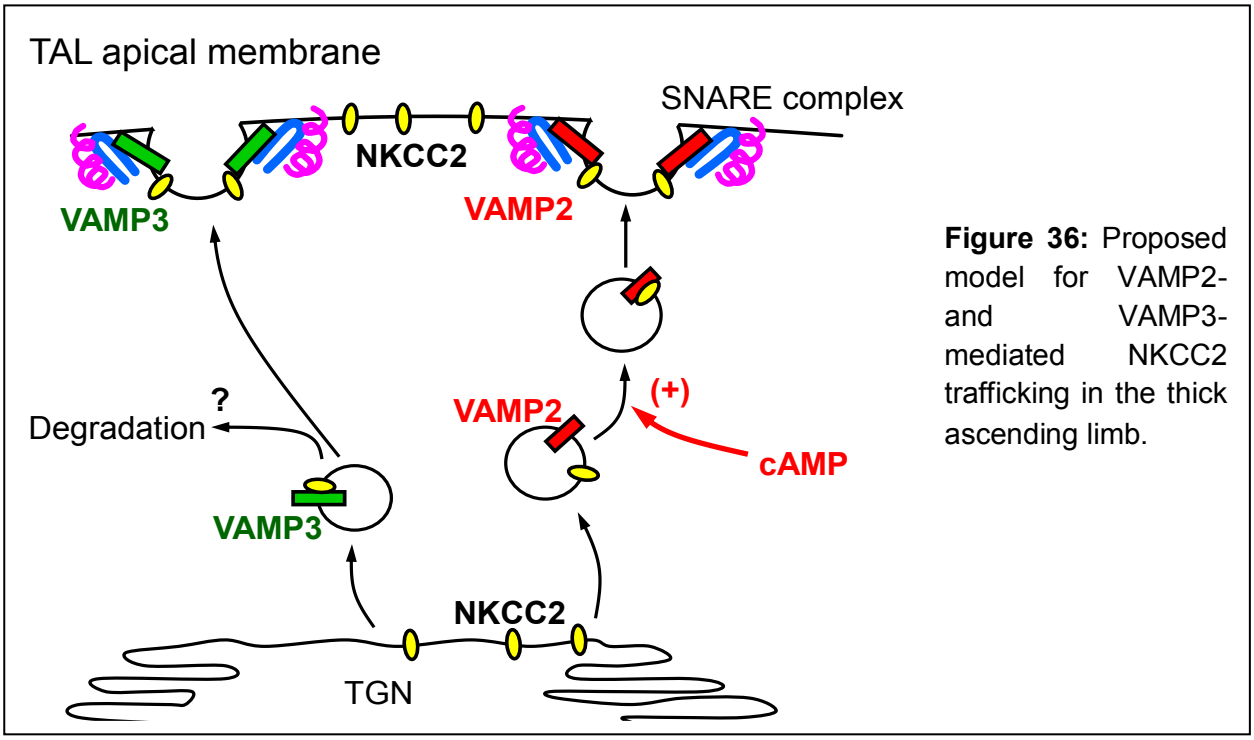

Figure 36: Proposed model for VAMP2- and VAMP3-mediated NKCC2 trafficking in the thick ascending limb.

Discussion

NKCC2 trafficking to the thick ascending limb surface is mediated by VAMPs

In the TAL, most NKCC2 is present in intracellular vesicles in the sub-apical space (76), while about 3-5% is present at the apical cell surface (14). However, this small proportion of surface NKCC2 mediates all of the NaCl absorption by the TAL and greatly contributes to control of blood pressure. Since NKCC2 is a big molecule and requires a number of post-translational modifications like glycosylation, it is highly likely that errors during the synthesis and maturation process produce defective molecules. It has been shown that defective maturation of NKCC2 retains the co-transporter in the endoplasmic

reticulum, preventing it from reaching the plasma membrane (194,195). Under this view, it is possible that excessive synthesis of NKCC2 is a mechanism adopted by the TAL cell to maintain accurate supply of the co-transporter at the cell surface.

There is a direct relationship between the abundance of NKCC2 in the apical surface and NaCl absorption by the TAL (14,24,46,167). Trafficking of NKCC2 to the apical membrane regulates NaCl absorption by the TAL (14,24). For instance, cAMP stimulates NaCl absorption in the TAL by enhancing NKCC2 exocytic delivery, augmenting steady-state surface NKCC2 levels (14,77). In addition, enhanced trafficking of NKCC2 is related to abnormally enhanced TAL NaCl reabsorption in animal models of hypertension (46), highlighting the relevance of this process in disease. The molecular mediators of NKCC2 trafficking are still poorly characterized. In this project we showed that several VAMP isoforms belonging to the SNARE family of membrane fusion proteins are expressed in the TAL. In addition to VAMP2 and 3, previously found in the TAL (14), we observed that VAMP7 and VAMP8 are also expressed in the TAL. However, VAMP7 and 8 are generally associated with endosomal trafficking, since they mediate vesicle-vesicle fusion (145,146). Therefore we studied VAMP2 and VAMP3, since most evidence indicates they mediate exocytic events involving fusion of intracellular vesicles with the plasma membrane (150,179,180,183,184). VAMP2 and VAMP3 are specifically cleaved by tetanus toxin (148,196-198). We have previously shown that tetanus toxin prevents cAMP-stimulated rise in steady-state surface NKCC2 expression (14). In the present study we showed that tetanus toxin also inhibits constitutive steady-state surface NKCC2 expression in the absence of cAMP. To differentiate between the roles of VAMP2 and VAMP3, we used a gene silencing approach *in vivo*. We studied whether VAMP2 and VAMP3 selectively mediate two different trafficking pathways for NKCC2 delivery to the TAL surface.

VAMP2 mediates cAMP-stimulated but not constitutive NKCC2 trafficking in thick ascending limbs

To study VAMP2, we developed a procedure to silence VAMP2 *in vivo via* adenovirus-mediated delivery of shRNAs. We found that silencing VAMP2 completely blocked cAMP-stimulated NKCC2 exocytic delivery without affecting constitutive NKCC2 delivery. Exocytic delivery along with endocytic retrieval contributes to maintaining steady-state surface NKCC2 expression. The inhibition of cAMP-stimulated exocytic delivery of NKCC2 we observed when silencing VAMP2 is expected to decrease steady-state surface NKCC2 expression. We tested this by surface biotinylation and indeed we found that silencing VAMP2 inhibited cAMP-stimulated steady-state surface NKCC2 by ≈50%. It is not clear why the magnitude of inhibition was smaller for cAMP-stimulated steady-state surface NKCC2. We do not think this is due to insufficient VAMP2 silencing. We achieved ≈70% specific reduction in VAMP2 protein expression and this was enough to completely block cAMP-stimulated NKCC2 exocytic delivery. A more likely explanation is that a fraction of the increase in steady-state surface NKCC2 caused by cAMP is due to inhibition of endocytosis. Since VAMPs mediate vesicle fusion, it may seem counterintuitive that they play a role in endocytosis. However, VAMPs also mediate vesicle-vesicle fusion between intracellular compartments. It has been shown that VAMP-mediated vesicle fusion is important for maturation of endocytic compartments in other cells (199-201). We cannot rule out the possibility that VAMP2 mediates endocytic retrieval of NKCC2. Our laboratory has previously shown that inhibition of endocytic retrieval of NKCC2 increases steady-state surface levels (78,79). However, it is not known whether cAMP inhibits endocytic retrieval of NKCC2 from the TAL surface and what the signaling mechanism may be. While studying this is of great interest, it is beyond the scope of our study.

In non-epithelial cells, VAMP2 has been associated with hormone-stimulated trafficking (151,170-172). In adipose and muscle cells, insulin stimulates translocation of GLUT4-carrying vesicles associated with VAMP2 (170,179,202,203). However, the role of VAMP2 in renal tissue is poorly understood. This is particularly difficult to address at the whole animal level, since VAMP2 knockout mice die *in utero* (180). The collecting duct is a nephron segment downstream from the TAL that expresses VAMP2 (137). In a collecting duct cell line, VAMP2 mediates constitutive as well as cAMP-stimulated aquaporin-2 translocation (106). To our knowledge, the data we present here are the first evidence that VAMP2 mediates cAMP-stimulated trafficking in native renal epithelial cells.

Hormonal stimulation of cAMP enhances NKCC2-mediated NaCl reabsorption in the TAL. We have previously shown that cAMP stimulates NKCC2 exocytic delivery *via* PKA (77). To rule out the possibility that silencing VAMP2 inhibits PKA signaling or activity thereby blocking NKCC2 exocytic delivery, we measured NKCC2 phosphorylation at NKCC2 Ser-126, which is a direct target of PKA (72). We found that despite blocking NKCC2 delivery, silencing VAMP2 did not decrease cAMP-stimulated phosphorylation of NKCC2 at Ser-126. Therefore, our data indicate that VAMP2 silencing does not affect overall PKA activity or the ability of PKA to phosphorylate NKCC2. These data suggest that VAMP2 is downstream from NKCC2 phosphorylation or is not required for the acute effect (20 min) of cAMP on exocytic delivery.

VAMP2 does not mediate constitutive trafficking of NKCC2 since we did not observe a decrease in baseline surface NKCC2 expression when we silenced VAMP2. According to our hypothesis, silencing VAMP2 should decrease the effect of cAMP signaling on surface NKCC2. However, it is likely that there is minimal cAMP production under baseline conditions. Previous reports indicate that nitric oxide and cGMP reduce surface NKCC2

and NKCC2 activity by decreasing intracellular cAMP concentration below baseline levels (15,24). This seems to contradict our proposed model since by silencing VAMP2 we would expect a decrease in NKCC2 trafficking due to reduction of cAMP signaling below baseline levels. However it is possible that reduction in cAMP and increases in cAMP mediate different signaling pathways. In fact, the PKA inhibitor H-89 does not decrease baseline steady-state surface NKCC2 expression in TALs (69,77). It is possible that PKA mediates cAMP stimulation of NKCC2 trafficking, but it does not maintain constitutive NKCC2 trafficking. The cAMP signaling pathway by which nitric oxide and cGMP decrease surface NKCC2 is still not clear. We propose that the VAMP2-dependent pathway for NKCC2 trafficking may be mediated by PKA, but it should not influence constitutive NKCC2 trafficking.

In conclusion, we have described for the first time a VAMP2-mediated pathway in renal epithelial cells that specifically mediates cAMP-stimulated but not constitutive NKCC2 trafficking.

VAMP3 mediates constitutive but not cAMP-stimulated NKCC2 trafficking in thick ascending limbs

Tetanus toxin cleaves VAMP2 and VAMP3 and we found that VAMP2 does not mediate constitutive NKCC2 trafficking. Thus, our data suggest that VAMP3 mediates constitutive NKCC2 trafficking. To directly address this, we silenced VAMP3 *in vivo* in TALs *via* adenoviruses-delivered shRNA. We achieved efficient and specific silencing, with almost 70% decrease in VAMP3 expression and no change in other VAMP isoforms. We observed that VAMP3 mediates constitutive exocytic delivery of NKCC2 and steady-state surface expression. In addition, VAMP3 does not mediate cAMP-stimulated exocytic delivery or steady-state surface NKCC2 expression, which we showed is mediated by

VAMP2. These observations were confirmed in VAMP3 knockout mice, which showed decreased constitutive surface NKCC2 expression. Our data indicate that the constitutive and cAMP-stimulated pathways are controlled by different sets of proteins. This is an interesting physiological concept, since each pathway could be controlled independently without interfering with one another. However, this does not seem to occur in every cell type. In neurons, ablation of the VAMP3 gene is compensated for by up regulation of VAMP2 and this rescues neurotransmitter release (184). This early observation gave rise to the idea that some VAMP isoforms may be functionally interchangeable and that is actually the case in some cell types (183,204) and *in vitro* (205,206). Several pieces of evidence from our observations indicate that for NKCC2 trafficking VAMP2 and VAMP3 do not compensate for each other and they are not interchangeable: First, we did not observe over expression of the opposite isoform in the TAL during silencing or during genetic deletion in VAMP3 knockout mice. Second, silencing VAMP2 caused a different effect than silencing VAMP3 on NKCC2 exocytic delivery. Third, the observation that VAMP3 is required for total NKCC2 protein expression while VAMP2 is not.

In adipose (151) and juxtaglomerular cells (169) there is evidence of physical segregation of VAMP2 and VAMP3 into two different compartments. Interestingly, in both cell types the VAMP2 positive pools are subjected to stimulation while the VAMP3 positive pools are not (169,170,179,202,203). In this dissertation we showed that VAMP2 and VAMP3 mediate different trafficking pathways. However, the possibility of physical separation between the two isoforms was not addressed in our study. This missing piece of information could provide insight into the molecular mechanism by which VAMP2 and VAMP3 mediate different pathways. However, in the present thesis we focused on the functional aspect as mediators of the final step that determines the amount of NKCC2 that

reaches the plasma membrane.

Considerations about the separate roles of VAMP2 and VAMP3 in NKCC2 trafficking

The concept that constitutive and cAMP-stimulated trafficking of NKCC2 is controlled by distinct proteins mediating two separate pathways can have important ramifications. The only NKCC2 inhibitors currently used in the clinic are loop diuretics like furosemide, bumetanide and torasemide. They are among the first choices for conditions like congestive heart failure and edema, when a dramatic reduction in extracellular volume is required. However, long term use to treat hypertension is currently limited due to the high potency of these drugs, which leads to severe side effects associated with the extreme loss of fluid and electrolytes. The problem with current NKCC2 inhibitors is that they do not discriminate between the constitutive and stimulated pathways. In this dissertation we took the first step in identifying the molecular components that mediate constitutive and cAMP-stimulated NKCC2 trafficking. Further characterization of additional components will lead to the identification of selective targets in each pathway. Targeted therapeutic intervention in one pathway could provide the benefits of an effective loop diuretic, while leaving the other pathway undisturbed could minimize the side effects.

One step closer towards the characterization of the VAMP2 and the VAMP3-mediated pathways is to determine whether they are associated with two separate NKCC2 pools. In other cells, VAMP2 and VAMP3 are physically segregated in two different vesicle populations (151,169). This can be of importance since preliminary experiments in our laboratory indicate that VAMP2 co-localizes with Rab-11 in TALs, a marker of recycling endosomes (unpublished). It is possible that the identity of the VAMP2 and VAMP3-positive compartments may be different, associated with the distinct roles in NKCC2 trafficking. However we did not address this further in our study.

It may be possible to purify vesicles carrying VAMP2 or VAMP3 from TALs by immunoprecipitation as performed previously in collecting duct preparations (137,152). Then it should be possible to determine whether these vesicles also express NKCC2 and the other VAMP isoform. Alternatively, co-localization of VAMP2 and VAMP3 in the same vesicles could be addressed by electron microscopy. We did not test whether VAMP2 and VAMP3 are localized in different NKCC2-positive compartments. However, this does not have to be necessarily the case since the two VAMP isoforms can be in the same vesicle and one of them can be inactive. For instance, in parotid acinar cells, VAMP2-mediated exocytosis of amylase granules is constitutively repressed by an inhibitory protein that binds the SNARE domain of VAMP2 (207). This inhibition is released after activation of the PKA pathway and amylase granules get exocytosed. Unfortunately, it was not studied whether amylase granules also contained other VAMP isoforms.

If NKCC2 is differentially associated with compartments of different molecular identity, this could be due to deliberate sorting of NKCC2 into two or more different intracellular pools. To our knowledge, there are no studies to this respect in the TAL. Alternatively, the NKCC2 compartments could change composition with time, in this way they may acquire a different VAMP isoform at a later stage in the maturation process. The age of vesicles in different cell systems has been monitored by using a special fluorescent protein that changes the emission wavelength from green to red as the vesicles age (208). In astrocytes, as VAMP2 vesicles age, they are sorted into two spatially separated populations (209). Similarly, secretory granules in chromafin cells are also segregated according to their age (210). Interestingly, some stimuli selectively produce the release of younger vesicles, whereas other stimuli preferentially cause the release of older vesicles. Whether this temporal segregation occurs in NKCC2 vesicles associated with VAMP2 or

VAMP3 is an interesting possibility that remains to be studied.

VAMP3 but not VAMP2 is required for normal NKCC2 expression

While silencing VAMP2 did not affect total NKCC2 expression, silencing VAMP3 decreased total NKCC2 by 45%. NKCC2 expression was also decreased by 66% in VAMP3 knockout mice. We concluded that VAMP3 but not VAMP2 is required for normal levels of NKCC2 protein expression. However, tetanus toxin did not decrease total NKCC2 expression in TALs. This is likely due to the shorter time required for treatment with tetanus toxin (50 minutes) compared to the three days required for VAMP3 silencing. We speculate that VAMP3 is required for maintenance of normal levels of NKCC2 in a process that takes longer than one hour.

There are at least three possibilities by which VAMP3 could regulate NKCC2 expression. First, VAMP3 could be required for NKCC2 gene expression. However, to our knowledge, there is no evidence in the literature implicating SNAREs as mediators of gene transcription and/or translation. Second, VAMP3 could be required for endosome maturation and when VAMP3 is silenced, NKCC2 maturation is interrupted. Traditionally, VAMP7 (145) and VAMP8 (146) have been considered the VAMPs involved in endosome maturation, with early reports indicating that VAMP3 does not play any role in intracellular endosome fusion events (211). However, increasing amounts of evidence indicate that VAMP3 mediates several vesicle-vesicle fusion processes in the endosomal pathway (212-215). Recent reports have found that VAMP3 mediates autophagosome maturation in cultured cell lines (199,214). We cannot discard this possibility based on our experimental design. The third possibility is that blockade of VAMP3 function prevents NKCC2 from reaching the plasma membrane and the molecules retained in the intracellular compartment are re-routed for degradation. Our data showing that VAMP3 silencing

completely prevents NKCC2 exocytic delivery suggest this may be the case. One way of addressing the role of VAMP3 in maintaining NKCC2 expression is by pulse-chase experiments that can be performed in TAL cultures. This would allow distinguishing between NKCC2 synthesis/maturation and degradation. If VAMP3 mediates NKCC2 synthesis or maturation, silencing VAMP3 would produce a decrease in the rate of appearance of either the mature or immature NKCC2 form in pulse-chase experiments. Alternatively, if preventing NKCC2 from reaching the cell surface when VAMP3 is silenced causes re-direction of NKCC2 to degradation, we would expect enhanced disappearance of labeled NKCC2 in pulse-chase experiments. Although these studies would provide a more complete picture of the mechanism we propose, they do not contribute to the focused hypothesis we tested here. We propose the pulse-chase studies as a future direction along with additional experiments to test a different hypothesis aimed to elucidate the mechanism for VAMP3-mediated NKCC2 trafficking.

The fact that constitutive NKCC2 exocytic delivery is almost completely inhibited when silencing VAMP3 also indicates that the decrease in steady-state surface NKCC2 is not just due to a proportional decrease in total NKCC2 protein expression. If this was the case, there is no reason to expect an 85% decrease in the rate of exocytic delivery as we observed.

NKCC2 co-localizes with VAMP2 and VAMP3 at apical surface clusters in thick ascending limbs

It is known that after vesicle fusion, VAMPs will remain inserted in the plasma membrane until they are retrieved to be re-used in another round of vesicle fusion (81,216). This allowed us to detect VAMP2 and VAMP3 at the plasma membrane after exocytosis has occurred. We used VAMP2-GFP and VAMP3-GFP constructs to study their surface

localization in primary cultures and whether they co-localized with surface NKCC2. We performed our studies in polarized TAL cultures that retain the characteristics of native tissue. This setup allowed us to easily access the apical membrane. We described for the first time that NKCC2 exhibits a heterogeneous distribution pattern at the apical membrane, being restricted to discrete micro domains or clusters. NKCC2 co-localized with VAMP2 and to a lesser extent with VAMP3 in those surface clusters.

The biological significance of this membrane compartmentalization is not clear. In hypocampal neurons, the Kv2.1 potassium channel is also distributed in clusters composed of non-conducting channels (217) that serve as platforms for exocytosis of Kv2.1 and Kv1.4 channels (218). Whether NKCC2 clusters conduct ions or serve as platforms for exocytosis in the TAL is an interesting possibility but it remains to be tested.

We observed that VAMP2 and VAMP3 are also distributed in apical micro domains. There is at least one previous report showing that in a cultured adrenal gland cell line, the site of exocytic vesicle fusion is highly dependent on the density of plasma membrane-resident SNAREs (syntaxin 1) (219). This prompted us to speculate that VAMP2- and VAMP3-mediated exocytosis and delivery of NKCC2 may occur at defined domains at the cell surface. An alternative interpretation for the clustered distribution of VAMP2 and VAMP3 is that once they reach the membrane, they undergo translational movement and coalesce into high density areas as described for other plasma membrane proteins (220-225). These high density areas can be sites of enhanced endocytosis (223,226-228). Translational mobility of transmembrane proteins in the plane of the membrane is determined by the cytoskeleton (222,229-231) and their interaction with lipids. VAMP2 and VAMP3 (232), as well as other SNAREs (233-235) have been reported to associate with the cytoskeleton in other cell types, suggesting they might be anchored or retained in a

micro domain after exocytosis.

Our co-localization analysis shows that NKCC2 shares an apical location with VAMP2 and VAMP3 at surface clusters. Whether VAMP2 and VAMP3 are present in the same or different clusters was not addressed in our study. Also, the resolution of confocal microscopy only allowed us to conclude that they share an apical location. Whether they are close enough at the clusters to physically interact with each other is not known. In neurons, VAMP2 interacts with potassium channels and this interaction modulates channel gating at the plasma membrane (236,159). This kind of regulation is not necessarily linked to trafficking, but it can be direct modulation of the intrinsic activity of the channel. Whether VAMPs directly regulate intrinsic NKCC2 activity was not addressed in this project.

Studying the dynamics of surface NKCC2, surface VAMP2 and VAMP3 micro domains is an interesting future direction in understanding the molecular regulation of NKCC2-mediated TAL function and renal physiology.

NKCC2 interacts with VAMP2 and VAMP3 in the thick ascending limb

We showed here that VAMP2 and VAMP3 mediate NKCC2 trafficking in TALs. To clarify how this may occur, we explored the possibility of protein-protein interaction between NKCC2 and these two VAMP isoforms. We showed by co-immunoprecipitation that VAMP2 and VAMP3 interact with NKCC2 in the TAL. To our knowledge, there are no reports in the literature of physical interaction between VAMP3 and non-SNARE proteins. However, in neurons VAMP2 physically interacts with P/Q-type Ca channels (158) and Kv2.1 potassium channels (159,160). The significance of these interactions is that they modulate channel activity. VAMP2 interacts with the cytoplasmic amino-terminus of Kv2.1, interfering with binding of a regulatory carboxy-terminal domain (236) and enhancing inactivation of channel gating (159). This effect is independent of channel trafficking to the

plasma membrane, suggesting that VAMPs may have a direct regulatory role not related with vesicle fusion. We showed that VAMP2 and VAMP3 mediate NKCC2 trafficking, but we cannot rule out a possible role as direct regulators of NKCC2 ion transport activity.

Here we also showed that VAMP2 interacts with a cytoplasmic domain in NKCC2. The functional significance of this was not directly addressed in our studies. However, this interaction region is a carboxy-terminal domain in NKCC2 previously shown to contain a signal that directs NKCC2 to the apical membrane (177). Also, this carboxy-terminal region has been previously reported to interact with MAL (163), a protein that inhibits NKCC2 endocytic retrieval, and with moesin (164), which enhances NKCC2 delivery to the surface. Both interacting proteins have the effect of increasing surface NKCC2 expression. This suggests that VAMP2 interaction with a targeting signal in NKCC2 may be important in regulated NKCC2 trafficking. It is known that incorrect targeting of NKCC2 to the wrong cellular localization has detrimental consequences for NKCC2-mediated NaCl transport (42,194). Another carboxy-terminal region, more proximal to the transmembrane portion of NKCC2, interacts with SCAMP2 (166) and Aldolase B (165). SCAMP2 inhibits NKCC2 exocytic delivery (166) and Aldolase B stimulates NKCC2 endocytosis (165) in cultured cells, both having the effect of decreasing the abundance of NKCC2 at the plasma membrane. Preliminary GST pull down experiments from our laboratory indicate that VAMP2 does not interact with the rest of the carboxy-terminus of NKCC2. However, the tree-dimensional structure of the whole NKCC2 molecule may have an influence in the interaction with VAMP2. This information may be overlooked when using fragments for GST pull downs. NKCC2-VAMP3 interaction presents a similar problem. We were not able to identify the region of NKCC2 that interacts with VAMP3 in our preliminary GST pull downs with the different NKCC2 fragments (not shown). There are three possibilities that

could explain this: 1) the interacting region falls in between the segments we used, 2) NKCC2 interaction with VAMP3 requires bigger portions or the whole NKCC2 molecule, 3) VAMP3 interacts with the transmembrane domains of NKCC2 (the amino- and carboxy-terminal portions of NKCC2 are cytoplasmic).

We propose that NKCC2 interactions with VAMP2 and VAMP3 may be part of the mechanism that regulates NKCC2 exocytic delivery to the TAL surface. However, we did not directly address the significance of these interactions. Although this goes beyond the scope of our hypothesis, addressing this is important to differentiate the roles of VAMP2 and VAMP3 in NKCC2 trafficking from a possible role in direct regulation of ion transport activity.

cAMP stimulates VAMP2 exocytosis, VAMP2-NKCC2 interaction and apical surface co-localization

We showed that VAMP2 mediates cAMP-stimulated NKCC2 trafficking. We also showed that VAMP2 reaches the apical surface and is located in clusters. Therefore we next tested whether cAMP enhances delivery of VAMP2 to the apical TAL surface. We found that cAMP almost doubled the rate of VAMP2 delivery to the TAL surface as tested by surface biotinylation. We also found by confocal microscopy that cAMP enhanced co-localization of surface NKCC2 with VAMP2 at apical membrane clusters. Interestingly, cAMP did not affect the clustered distribution pattern of surface VAMP2 or NKCC2. This may be an indication that VAMP2 exocytosis occurs preferentially at these clusters. In order to confirm this directly, real time monitoring of active VAMP2 exocytosis may be required.

In the TAL, cAMP stimulates NKCC2-mediated NaCl absorption *via* PKA (16,18). Also, we have previously shown that cAMP stimulates NKCC2 exocytic delivery *via* PKA (74,77,80). Here we found that stimulation of PKA with a cAMP analogue enhanced

NKCC2 interaction with VAMP2. Since we are not able to determine whether NKCC2-VAMP2 interaction occurs in intracellular vesicles or at the plasma membrane, we should consider two possible scenarios: 1) PKA stimulates NKCC2-VAMP2 interaction in intracellular vesicles and this enhances NKCC2 exocytic delivery. 2) PKA stimulates NKCC2-VAMP2 interaction at the plasma membrane and this stimulates intrinsic NKCC2 ion transport activity. We showed that VAMP2 mediates cAMP-stimulated NKCC2 trafficking, so we favor the first scenario. However, we cannot rule out the second scenario since we did not test whether VAMP2 interaction with NKCC2 occurs at the plasma membrane or whether this interaction regulates NKCC2 ion transport capabilities. In any case, these two scenarios are not necessarily mutually exclusive.

Whether the SNARE machinery and VAMP2 are targets of PKA-mediated phosphorylation in the TAL is not known. We have preliminary data indicating that PKA phosphorylates VAMP2 in TALs (unpublished). However, the mechanism and site of phosphorylation is unclear since VAMP2 does not have consensus PKA phosphorylation sites. VAMP2 stimulation by PKA could be mediated by intermediate proteins or additional downstream kinases. In parotid acinar cells, VAMP2 mediates PKA-stimulated granule exocytosis *via* interaction with a non-identified intermediate protein (207). These intermediate proteins can be other members of the SNARE machinery. For instance, syntaxin 4 (237) and SNAP-25 (238) are known to be phosphorylated by PKA *in vitro* and in other cells. Alternatively, SNARE-accessory proteins that regulate SNARE complex formation can be the target of PKA stimulation. In other cells, SNARE-associated proteins, including snapin (239), tomosyn (240), complexin (109) and Munc18 (241) are targets of the PKA pathway. From our studies, we cannot rule out the involvement of SNARE regulatory proteins in VAMP2-mediated NKCC2 trafficking and their phosphorylation by

PKA as a possible mechanism.

In general, how phosphorylation influences the interaction of SNAREs with transmembrane transporters is not well characterized. To our knowledge, there are no studies addressing the role of PKA in regulating SNAREs interaction with ion transporters. On the other hand, phosphorylation by PKC and Ca/ calmodulin-dependent kinase II regulates interaction of SNARE proteins and neuronal Ca channels (156). In a different report, Zhu *et al.* (242) showed that Glycogen synthase kinase-3 (GSK-3) inhibits Ca channels in neurons and synaptic vesicle release. GSK-3 achieves this *via* phosphorylation-mediated inhibition of interactions between the Ca channels and SNARE components, and also by inhibiting the formation of the SNARE complex. The enhanced NKCC2 interaction with VAMP2 we observed in the presence of PKA stimulation is likely to be part of the mechanism by which PKA stimulates NKCC2. Whether this mechanism involves stimulation of NKCC2 trafficking or direct activation of NKCC2-mediated ion transport is still to be determined.

Direct phosphorylation of NKCC2 itself by PKA could also be the mechanism by which PKA enhances NKCC2-VAMP2 interaction. NKCC2 phosphorylation at Ser-126 by PKA is important for stimulation of NKCC2 activity (73). We showed that silencing VAMP2 did not affect NKCC2 phosphorylation at Ser-126, indicating that VAMP2 functions downstream Ser-126 phosphorylation or they are part of different pathways. There is another serine in position 874 in NKCC2 that is phosphorylated by PKA (72). In addition, there are at least two more PKA consensus sites within the NKCC2 sequence that have never been confirmed empirically. Any of the PKA phosphorylation sites described has the potential of enhancing NKCC2 interaction with VAMP2.

Taken together, our data indicate that cAMP enhances VAMP2-NKCC2 co-

localization at discrete micro domains in the apical membrane of TAL cells. Similar to NKCC2, cAMP stimulates VAMP2 exocytic delivery to the apical surface and stimulation of PKA enhances VAMP2-NKCC2 interaction. All this, together with our previous observation that NKCC2 and VAMP2 interact *via* a carboxy-terminal region in NKCC2 that contains an apical localization signal, opens the possibility that this may be part of the mechanism by which cAMP stimulates NKCC2 delivery to the cell surface.

Renal phenotype of VAMP3 knockout mice

Since VAMP3 mediates constitutive trafficking of NKCC2 to the apical surface of TALs, we would expect VAMP3 knockout mice to exhibit a renal phenotype consistent with enhanced excretion of water and electrolytes. To further study the role of VAMP3 in water and electrolyte homeostasis, we compared urinary excretion between wild-type and VAMP3 knockout mice. We observed that VAMP3 knockout mice produced more diluted urine compared to wild-types under normal water intake indicating a decreased ability of these mice to concentrate urine. They also excreted more Ca, consistent with decreased NKCC2-mediated NaCl reabsorption. However, we did not observe major loss of Na, Cl or K in urine. To our knowledge, the only other instance in which the role of a SNARE in renal physiology has been addressed at the whole animal level has been in a study performed in VAMP8 knockouts (121). VAMP8 knockout mice exhibit polyuria and decreased vasopressin-enhanced urinary osmolality without a change in Na, K, Cl and Ca excretion. This effect was attributed to defective aquaporin-2 translocation in the collecting duct but other nephron segments were not studied. The phenotype in VAMP8 knockout mice is more severe than the one we observed in VAMP3 knockouts. It is likely that the various SNAREs have a different pattern of expression along the nephron such that they regulate different ion and water transporters. However, this study and ours emphasize the

importance of VAMPs in the ability of the kidney to reabsorb water and salt.

VAMP3 knockout mice excreted a urine volume that was 21% higher than wild-types. However, it did not reach statistical significance. Whether this increase is enough to cause the observed decrease in urine osmolality is not clear. We only observed enhanced excretion of Ca, whose contribution to urine osmolality is minimal compared to other ions. We did not observe any change in excretion of Na, Cl and K. However, we did not measure other osmolytes like urea, which can account for the lower urine osmolality in VAMP3 knockout mice.

Overall, the renal phenotype exhibited by VAMP3 knockouts is mild. This is not surprising, since these mice have previously been shown to display normal constitutive and insulin-stimulated trafficking (186), normal granule content release from platelets (188), normal phagocytosis in macrophages (187) and their overall health we and others observed is good. It is possible that other VAMPs compensate for the loss of VAMP3. There is evidence that VAMP2 compensates for lost VAMP3 function in nerve cells in the VAMP3 knockout (184). The opposite does not occur during VAMP2 genetic deletion, which is lethal (180). In the TAL we observed little evidence of compensation by VAMP2. However, silencing VAMP3 by ≈70% decreased steady-state surface NKCC2 by 43% and genetically deleting VAMP3 decreased steady-state surface NKCC2 by 46%, suggesting that another VAMP different than VAMP2 could compensate part of VAMP3 function in knockout mice.

Fluid and Ca loss stimulate the cAMP pathway in the distal nephron by enhancing circulating vasopressin and parathyroid hormone (PTH) respectively. Stimulation of cAMP in the TAL would increase VAMP2-mediated NKCC2 trafficking and activity. We discuss the possible compensatory mechanisms for water and ion handling separately (Figure 37).

- Water handling by the kidney in VAMP3 knockout mice

VAMP3 knockout mice excreted a slightly higher urine volume (1.62 ± 0.16 *vs.* 1.33 ± 0.12 ml/24h) and produced less concentrated urine. However the polyuria was not as severe as in VAMP8 (121) or NKCC2 (40) knockouts. We propose that the water loss in VAMP3 knockout is minimized by enhanced circulating vasopressin (Figure 37). The baseline situation is represented in Figure 37a. Normal NKCC2-mediated Na absorption by the TAL contributes to building up the hyperosmotic interstitium for water absorption by collecting ducts, preventing water loss in urine. In VAMP3 knockouts, NKCC2 activity is decreased, reducing Na absorption and decreasing the renal interstitial osmolality required for water reabsorption (Figure 37b). This results in water loss and increased blood

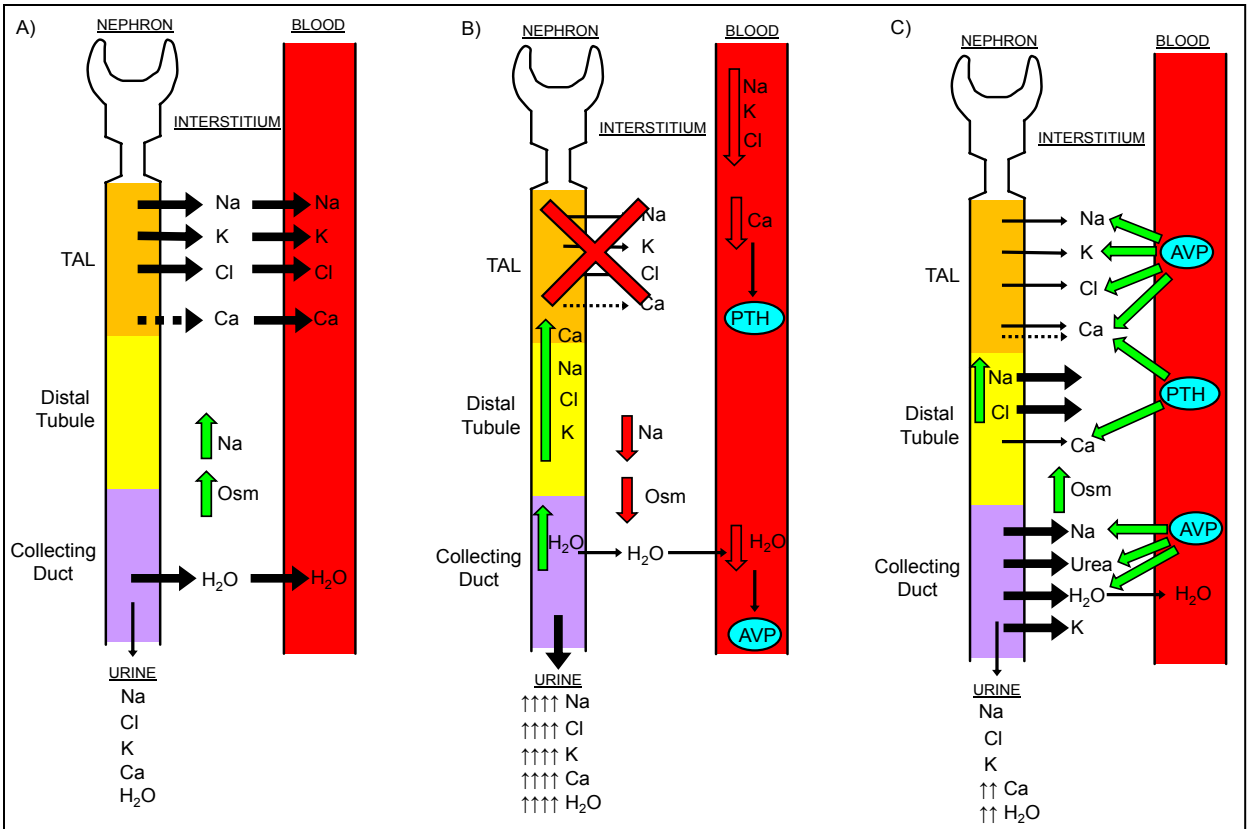

Figure 37: Proposed mechanism for renal phenotype of VAMP3 knockout mice: A) Normal condition. B) Impaired TAL ion transport in VAMP3 knockout mice. C) Compensatory adjustments in response to decreased baseline NKCC2 activity. AVP: Arginine-Vasopressin. PTH: Parathyroid hormone. Osm: osmolality. Dotted arrow = paracellular pathway. Solid arrow = trans-cellular pathway.

osmolality which stimulates vasopressin release to the circulation from the pituitary gland. Vasopressin activates the cAMP pathway in the TAL and stimulates NKCC2 *via* VAMP2 (Figure 37c). In addition, vasopressin enhances water reabsorption in the collecting duct by stimulating the water channel aquaporin-2 (243-245) and enhances urea transport (246) which contributes to the hyperosmotic medulla. All this would tend to minimize water loss in VAMP3 knockout mice. We did not measure circulating vasopressin in VAMP3 knockout mice, however we would expect it to be increased. In addition, increased circulating PTH would also activate NKCC2 *via* cAMP (56,63,65). We expect circulating PTH to be increased due to the Ca loss and we will discuss the role of PTH next.

- Urinary Ca handling in VAMP3 knockout mice

We found enhanced urinary Ca excretion in VAMP3 knockouts, which is consistent with decreased NKCC2 activity since Ca loss is also observed in NKCC2 knockout mice (40) and also in patients with Bartter syndrome (32,34-36,247-250), a pathology caused by loss of NKCC2 function (27,31,35). The major site for renal Ca reabsorption is the proximal tubule. However the fine-tuning of Ca reabsorption occurs in the TAL (Figure 37a). Ca is reabsorbed in the TAL *via* the paracellular pathway, in a process driven by the lumen-positive electric potential created by the joint action of NKCC2 and the potassium channel ROMK (Figure 2). If VAMP3 knockouts have decreased NKCC2 activity, the driving force for Ca absorption is decreased, producing Ca loss in the urine (Figure 37b). The ensuing Ca depletion in the blood stimulates PTH release from the parathyroid glands. PTH stimulates NKCC2 and ROMK *via* cAMP, restoring the electrical gradient but also stimulates the paracellular pathway for Ca absorption in the TAL and Ca reabsorption across the distal tubule (Figure 37c). We expect all these compensatory mechanisms are active in VAMP3 knockout mice, thereby minimizing urinary Ca loss.

- Urinary Na, Cl and K handling in VAMP3 knockout mice

Normally between 25-30% of the Na and Cl filtered in the glomerulus is reabsorbed in the TAL. NKCC2 is a major contributor to this process, preventing loss in the urine (Figure 37a). If VAMP3 knockouts have decreased NKCC2 activity, we would expect increased Na, Cl and K excretion (Figure 37b). However, VAMP3 knockouts did not experience any major wasting of Na, K and Cl in the urine under baseline conditions. It is likely that other nephron segments distal to the TAL enhance Na, K, and Cl transport to counteract any major defect in the TAL (Figure 37c). It is known that enhanced luminal Na delivery to the distal tubule enhances NaCl reabsorption *via* the thiazide-sensitive Na/Cl co-transporter NCC (251,252). Also, enhanced circulating vasopressin can stimulate Na reabsorption by NCC and in the collecting duct *via* the epithelial Na channel ENaC (253,254). In addition, NKCC2 function could be partially restored *via* the cAMP-VAMP2 pathway as consequence of the increased circulating vasopressin and PTH mentioned above. Finally, the collecting duct also contributes to K excretion by balancing secretion and reabsorption. All these actions could contribute to minimize Na, Cl and K loss in VAMP3 knockout mice.

Since we did not observe any difference in baseline Na, Cl and K excretion in VAMP3 knockout mice, we next tested whether they could adjust excretion of these ions when challenged to water deprivation. It is known that water restriction produces an increase in ion excretion in many mammal species (255-260). This response is believed to limit the increase in plasma osmolality caused by the limited fluid supply, which can be life threatening. A known mediator of this response is the hormone oxytocin (191), which is released from the neurohypophysis in response to hyperosmolality (261). It has been proposed that oxytocin mediates its natriuretic and kaliuretic effect by stimulating release of

atrial natriuretic peptide (ANP) from the heart (262). There are oxytocin receptors in the heart (192) and infusion of oxytocin in isolated hearts produce release of ANP (192). ANP can enhance ion excretion by increasing glomerular filtration rate or by directly inhibiting ion reabsorption by the nephron. In addition, oxytocin itself is believed to synergize with ANP to inhibit tubular ion reabsorption *via* cGMP (263). The concerted actions of ANP and oxytocin cause natriuresis and kaliuresis independently of vasopressin (189,190), which is also released to the circulation in response to water deprivation.

When mice were water-deprived for 24 hours Na, K and Cl excretion augmented in both strains. However, VAMP3 knockouts excreted more Na, Cl and K than wild-types under water deprivation. This indicates that when challenged to water restriction stress, renal function does not adjust completely or it takes longer to adjust. This response is expected according to the model we propose. If there is compensation by other nephron segments more distal to the TAL, excretion of Na, Cl and K would be normal in VAMP3 knockouts under baseline conditions. When water-deprived, the inhibitory actions of ANP in ion transport in more distal nephron segments would override the initial compensation. This would allow us to observe a defect in TAL ion transport as excessive excretion of Na, Cl and K.

Another way of appreciating a renal defect in VAMP3 knockout mice, is by limiting the amount of Na in the diet. We achieved this by switching the animals to a low-Na diet. Under this condition, the kidneys would adjust to excrete less Na in an attempt to retain the limited Na supply in the diet. We observed that urinary Na excretion was not decreased as much in VAMP3 knockout mice as in wild-type mice after 24 hours in low-Na diet, but after 48 hours excretion of Na was comparable in both strains. This indicates that in VAMP3 knockouts renal function takes longer to adjust to limit Na excretion in the urine when

challenged to a condition like low-salt diet, when Na intake is limited. They eventually are successful in limiting Na excretion, otherwise this wasting of Na could be life-threatening. This adjustment most likely occurs *via* the compensatory mechanisms described before.

Altogether, our data indicate that VAMP3 knockout mice have decreased renal function, which limits their ability to concentrate the urine and to properly excrete electrolytes under baseline conditions and after challenges that demand homeostatic adjustments of water an ion balance. To our knowledge this is the first phenotype ever described for VAMP3 knockout mice, emphasizing the importance of VAMP3 in renal function.

Decreased arterial pressure in VAMP3 knockout mice

Although VAMP3 shows mild fluid and electrolyte loss in urine, blood pressure should be fully compensated by activation of the renin-angiotensin-aldosterone system. However, we observed a significantly lower systolic blood pressure (-19 mmHg) compared to wild-type mice. NKCC2 mutations in humans also decrease blood pressure (32-36,41,42). Our data indicate that VAMP3 plays an important role in maintaining normal blood pressure. The mechanism by which this occurs is unclear but may involve the role of VAMP3 in NKCC2-mediated NaCl absorption or a role of VAMP3 in regulation of other renal transporters. It has been suggested that VAMP3 participates in aquaporin-2 trafficking to the collecting duct membrane (106,147). In addition, VAMP3 can be important for endocrine control of blood pressure. It is known that VAMP3 does not mediate baseline or stimulated renin release from juxtaglomerular cells (169). However other components of the renin-angiotensin-aldosterone system may require VAMP3. For instance, angiotensinogen release from liver cells or aldosterone production from the adrenal cortex. Finally, VAMP3 may regulate vascular reactivity or neural control of arterial tone. To our

knowledge the role of VAMP3 in cardiovascular function and blood pressure has not been addressed.

Strengths and limitations of the study

Strengths: We performed all our experiments in native TALs or in primary culture of TALs which retain the characteristics of the native tissue. We consider that this approach enhances the significance of our findings. In addition, we described a mechanism that regulates NKCC2 trafficking at the molecular level and then tested the role of the major components of this mechanism at the whole animal level. We developed new techniques to silence genes *in vivo* and took advantage of a knockout model. All these approaches increase the translational relevance of the mechanisms we described to human and animal physiology.

Despite the differences that may exist between species, the underlying basic principles in cell biology, renal physiology and TAL function are well conserved among mammals. We believe the knowledge we gathered in this dissertation project is useful as first evidence of an important mechanism with potential implications in renal physiology and hypertension.

Limitations: There is a general limitation with any study using animal models as a representation of human physiology. However, the kind of analysis we conducted here would have been impossible to perform in human subjects.

Among the specific limitations concerning the current study we could mention:

1. Most of the experiments were performed in medullary TAL suspensions, which is an enriched sample but not completely pure. However, 90-95 % of the tubules are TALs and the rest is composed of other nephron segments and blood vessels. Nevertheless, NKCC2 is expressed exclusively in the TAL, making contamination irrelevant in this case since we

used a specific NKCC2 antibody. Regarding other proteins, we consider a suspension which is >90% TALs acceptable as a representative sample to measure protein expression.

2. The studies performed in primary cell culture isolate TAL cells from their native environment in the renal medulla. Any response we observed here is devoid of the context found *in vivo* and may not represent the natural condition. However, this was the only methodology available to us to gain access to the apical membrane after heterologous expression of VAMPs. On the other hand, these are more controlled conditions that eliminate confounding factors found in the complex environment *in vivo*.

3. We used forskolin + IBMX to maximally stimulate intracellular cAMP, since in our hands this is the most potent stimulus for NKCC2 trafficking. However, we recognize that this condition is not physiological and could only represent the convergence of more than one signaling pathway that uses cAMP as second messenger.

4. The co-immunoprecipitation protocols allowed us to determine that NKCC2 interacts with VAMP2 and VAMP3. However, we did not determine whether this interaction is direct or *via* intermediate proteins, since NKCC2 and VAMPs could precipitate as part of a larger protein complex.

5. We determined that cAMP stimulates NKCC2-VAMP2 interaction. However, we recognize that VAMP2 mediates exocytosis by inducing the formation of a SNARE complex. We have yet not identified the SNARE partners of VAMP2 that mediate NKCC2 trafficking. This goes beyond the scope of the hypothesis tested in this study.

6. VAMP2 and VAMP3 are very likely to be involved in delivery of other plasma membrane proteins beside NKCC2 in the TAL. This could have an indirect effect on NKCC2 trafficking. However, we found direct evidence of NKCC2 interaction and co-localization with VAMP2 and VAMP3 in the TAL, suggesting they are very likely to play a

direct role. By no means is this an indication that such a role is exclusive for NKCC2, and that is not the way we formulated our hypothesis or interpreted our results. It is very likely that VAMP2 or VAMP3 mediate trafficking of other components of the ion absorption machinery in the TAL, which include NHE3, ROMK, KCC, Na-K ATPase or Cl channels. We could not cover all of them in our study. Altering the trafficking of any of these components will most likely change the intracellular concentration of ions. It is known that decreased intracellular Cl stimulates NKCC2 activity (264), and this is likely due to enhanced ion transport instead of enhanced trafficking. We do not think that ion transport influences NKCC2 trafficking since unpublished experiments from our laboratory indicate that furosemide, which blocks ion transport by competing for the Cl binding site in NKCC2, does not affect surface NKCC2 expression.

We consider that testing whether VAMP2 and VAMP3 mediate trafficking of other membrane transporters in the TAL and how this may influence NKCC2 trafficking is a separate hypothesis that requires a different experimental design.

7. We measured renal function and blood pressure in global VAMP3 knockout mice. In order to address whether VAMP3 expressed specifically in the TAL mediates the effects we observed, a different model with TAL-targeted VAMP3 deletion is necessary. Although we believe our studies provide the first observation that VAMP3 is important for renal function and blood pressure, this does not mean VAMP3 in the TAL is exclusively responsible for the effects we found.

8. We were not able to study the role of VAMP2 deletion in TALs. Global VAMP2 knockouts are not viable. In order to address the role of VAMP2 in renal physiology, tissue-specific deletion of VAMP2, which is not lethal, would be required.

Perspectives

About 30% of Americans are hypertensive (265), costing the nation billions of dollars in healthcare costs and lost productivity (266). Hypertension is a major risk factor for cardiovascular disease, the number one cause of death worldwide (267) and in the United States (268). About 20% of hypertensive patients do not respond to current treatments (269), highlighting the need to further investigate the mechanisms of hypertension. The kidneys reduce extracellular fluid volume by enhancing sodium and water excretion in response to increases in arterial pressure (270), playing a central role in controlling blood pressure. In this dissertation we have described a molecular mechanism that regulates NKCC2, a key component of the machinery for NaCl reabsorption by the TAL, a nephron segment that has major influence on blood pressure. The only known inhibitors of NKCC2 are the loop diuretics furosemide, bumetanide and torasemide, with similar mechanism of action. However, their use is restricted to cases like congestive heart failure and edema in which a rapid and dramatic decrease in extracellular fluid volume is required. Despite the promise of drugs targeting NKCC2 to treat hypertension, the long-term use of loop diuretics is limited by their side effects, which include severe dehydration, potassium and calcium loss, cataracts and diminished hearing (271-276).

Here we propose a mechanism that regulates NKCC2 *via* the vesicle fusion proteins VAMP2 and VAMP3, whose genes overlap with several quantitative trait loci (QTL) in hypertensive rat strains. According to the Rat Genome Database, VAMP2 (chromosome 10) is present in 12 blood pressure QTL and VAMP3 (chromosome 5) is present in 5. The only known agents that target SNAREs are the several serotypes of botulinum toxin which cleave target membrane-SNAREs and VAMPs, and tetanus toxin which cleaves VAMP2 and VAMP3. More recently, small molecule inhibitors that interfere with the SNARE

complex formation have been developed (277). However, since SNAREs are ubiquitous, the therapeutic use of these drugs will be limited until a way to direct them to specific tissues is found. In this dissertation we found that different VAMPs control two distinct pathways in NKCC2 regulation. Further knowledge into the details of these independent pathways will lead to the eventual development of more precise and effective diuretics to treat hypertension. Being able to intervene selectively in a particular regulatory pathway without interfering with other pathways is appealing since the beneficial effects of a loop diuretic could be achieved with minimal side effects.

CHAPTER 5

GENERAL METHODS

Animals used in the study

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and were performed according to the committee's guidelines. We used male Sprague-Dawley rats weighing 130g, 18-week-old C57BL/6J mice and VAMP3 knockout mice on a C57BL/6J background. Rats were purchased from Charles River Breeding Laboratories (Wilmington, MA) and wild-type mice from Jackson Laboratory (Bar Harbor, ME). VAMP3 knockout mice were generated by the laboratory of Dr. Jeffrey Pessin (186) and generously shared with us. The VAMP3 gene was disrupted by deleting exons 2 and 3. Animals were fed a diet containing 0.22% sodium and 1.1% potassium (Purina, Richmond, IN) and had free access to water *ad libitum*.

Antibodies

The anti-NKCC2 antibody was raised in chicken by Lofstrand Labs Limited (Gaithersburg, MD). It recognizes amino acids 33–55 of the amino terminus of rat NKCC2 as does the L-320 antibody by Kim *et al.* (173). The mouse anti-GAPDH antibody was from Chemicon (Temecula, CA). The mouse anti-VAMP2 and rabbit anti-VAMP8 antibodies were purchased from Synaptic Systems (Goettingen, Germany). The rabbit anti-VAMP3 and mouse anti-VAMP7 antibodies were from Abcam (Cambridge, MA). The rabbit anti-phospho-serine 126 NKCC2 antibody was a generous gift from Dr. Mark Knepper (72). The HRP-conjugated secondary anti-mouse and anti-rabbit antibodies were obtained from GE Healthcare Life Sciences (Piscataway, NJ). The HRP-conjugated secondary anti-chicken IgG was from Gallus Immunotech (Fergus, ON, Canada). The rabbit NKCC2 antibody for immunoprecipitation was obtained from EMD Millipore (Billerica,

MA) and recognizes the carboxy-terminus of NKCC2. The exofacial NKCC2 antibody was developed by us in rabbit (24) and recognizes an epitope in the extracellular loop between transmembrane segments 5 and 6 in rat NKCC2. The Alexa Fluor 488- conjugated anti-GFP antibody and the Alexa Fluor 568-conjugated secondary antibody were from Life Technologies (Grand Island, NY). The mouse anti-GFP antibody for Western blot was from Clontech (Mountain View, CA).

Reagents

Reagents for steady-state surface biotinylation and exocytic delivery, and proteinG/proteinA-coupled agarose beads were from ThermoFisher Scientific (Waltham, MA). Forskolin and IBMX were from Sigma-Aldrich (St. Louis, MO). The glutathione-coupled sepharose beads were from Roche (Basel, Switzerland). N^6-Benzoyl-cAMP was purchased from BioLog (Bremen, Germany). Tetanus toxin was from List Biological Laboratories (Campbell, CA). The glutathione S-transferase (GST) expression vector was pGEX-6P-1 from GE Healthcare Life Sciences. The VQ Ad5CMV K-NpA adenovector was from Viraquest (North Liberty, IA). The VAMP2-eGFP and VAMP3-eGFP constructs were generously provided by Dr. Regazzi (179).

Medullary thick ascending limb suspensions

Suspensions of outer medullary TALs were obtained from rats according to a standard protocol used routinely in our laboratory (20). Briefly, rats were anesthetized with ketamine (100 mg/kg body wt ip) and xylazine (20 mg/kg body wt ip). Kidneys were perfused through the aorta with 0.1% collagenase in O_2-saturated HEPES-buffered perfusion solution. The outer medulla was dissected, minced and incubated at 37°C for 30 min with 0.1% collagenase. The composition of the HEPES-buffered perfusion solution was 135 mM NaCl; 10 mM HEPES; 4 mM KCl; 1.5 mM NaH_2PO_4; 1.2 mM $MgSO_4$; 1 mM

Ca-lactate$_2$; 5 mM alanine; 5.5 mM glucose; 1 mM Na_3-citrate; *p*H 7.4.

Primary culture of medullary thick ascending limbs

Individual TAL cells were obtained from rat medullary TAL suspensions after digesting the tubules with collagenase (0.1%), trypsin (0.25%) and DNAse (0.0021%). Single TAL cells were isolated by centrifugation at 41,300 x *g* in a 35% Percoll gradient, and seeded in Trans-well permeable supports (Corning, Tewksbury, MA) coated with basement membrane extract (Trevigen, Gaithersburg, MD). Cells were grown in DMEM medium supplemented with 1% fetal bovine serum and insulin-transferrin-selenium (Life Technologies) at 37ºC and 5% CO_2.

Western blot

TAL suspensions were lysed in buffer containing 150 mM NaCl, 50 mM HEPES (*p*H 7.8), 5 mM EDTA, 1% Triton X-100, 0.2% SDS and protease inhibitors (benzamidine, leupeptin, aprotinin, chymostatin and pepstatin-A, from Sigma-Aldrich). When measuring phosphorylation, the lysis buffer also contained PhosSTOP phosphatase inhibitors (Roche). Protein samples were run by electrophoresis in 6% SDS-polyacrylamide gels to detect NKCC2 and in 5% stacking- 12% resolving gels to detect VAMPs. Proteins were transferred to PVDF membranes overnight and the next day blocked in 5% milk in TBS-T buffer for one hour at room temperature. Next, membranes were incubated with the primary antibody for one hour at room temperature at the following dilutions: 1/700 (NKCC2), 1/2000 (p-Ser126-NKCC2), 1/2000 (VAMP2), 1/30,000 (VAMP3), 1/500 (VAMP7), 1/50,000 (GAPDH). Membranes were then incubated with the corresponding HRP-conjugated secondary antibody at 1/7000 (chicken) or 1/5000 (mouse and rabbit) for one hour at room temperature and developed by chemiluminescence.

***In vivo* gene silencing**

Construction of adenoviral vectors

The target sequence for the VAMP2 rat gene was 5'-GCTCAAGCGCAAATACTGG-3' and for VAMP3 was 5'-GGATCTTCTTCGAGACTTT-3'. Nude small interfering RNAs (siRNA) were synthesized with the Silencer siRNA construction kit from Applied Biosystems (Carlsbad, CA) and tested *in vitro* in NRK-52E cells from the American Type Culture Collection (Manassas, VA). Sense and antisense sequences spaced by a loop sequence (TTCAAGAGA) were sub-cloned between the 5′ AflII and 3′ SpeI sites in the Adenovector-pMIGHTY (Viraquest) to produce adenoviral particles coding short hairpin RNAs (shRNA). Adenoviral particles were assembled by Viraquest. A control construct coding a non-targeting sequence (5′-TTCTCCGAACGTGTCACGT-3′) was produced in the same way. The adenoviruses were tested again in NRK-52E cells at 100 plaque forming units (PFU) per cell. Expression of VAMP2, VAMP3, VAMP7, VAMP8 and GAPDH was monitored by Western blot.

In vivo transduction of TALs

Adenoviruses were injected into the renal outer medulla in rats as described previously (175). Briefly, rats were anesthetized with ketamine and xylazine and a small incision was made in the left flank to expose the kidney. The renal artery and vein were clamped to stop blood flow. Adenoviruses carrying the shRNAs were given a series of five injections (one minute each), with a pre-measured 30-gauge needle connected to a nanoliter syringe pump at 20 µl/min. After 8 minutes, the clamp was released, the kidney returned to the abdominal cavity and the incision was closed. The procedure was then repeated in the right kidney of the same rat but using the control shRNA sequence.

Biotinylation of steady-state surface NKCC2 in thick ascending limb suspensions

Medullary TAL suspensions were obtained from rats, equilibrated at 37°C for 15 min and then treated with either vehicle or forskolin (20 μM) plus IBMX (0.5 mM) for 30 min. TALs were biotinylated at 4°C with membrane-impermeant NHS-SS-biotin (0.9 mg/ml), washed and lysed in the presence of protease inhibitors. Biotinylated proteins were separated with streptavidin-coated beads overnight at 4°C and eluted by boiling in Laemmli loading buffer containing dithiothreitol (DTT) and β-mercaptoethanol. NKCC2 and GAPDH were detected by Western blot. In every experiment, we monitored total NKCC2 expression and absence of intracellular biotinylation by examination of GAPDH expression in the surface fraction.

Exocytic delivery of surface proteins

NKCC2 exocytic delivery

TAL suspensions were incubated with membrane-impermeant NHS-acetate (2 mg/ml) at *p*H 7.8 and 4°C for one hour to mask surface sites accessible to NHS-SS-biotin. Fresh NHS-acetate was added every 15 min. Forskolin and IBMX were added at 4°C and samples warmed to 37°C for 30 min. TALs were then cooled and newly inserted NKCC2 biotinylated with NHS-SS-biotin. he efficiency of NHS-acetate masking for NKCC2 was calculated in every experiment from the difference between a TAL aliquot that was not masked with NHS-acetate (100% basal surface NKCC2) and an aliquot that was masked at 4°C but never warmed to 37°C (0 time-point). The difference between these two samples represents the NHS-acetate-masked surface NKCC2 fraction, which was used to express exocytic delivery over time.

VAMP2-eGFP exocytic delivery

Primary cultures of TALs were obtained from rats and transfected with VAMP2-

eGFP *via* adenoviruses. Twenty four hours after transfection, cells were treated with vehicle or forskolin (20 μM) plus IBMX (0.5 mM). The GFP portion of VAMP2-eGFP faces the extracellular space and is accessible for biotinylation. We measured exocytic delivery by masking with NHS-acetate and biotinylating newly delivered VAMP2-eGFP in the same way as described for NKCC2. Surface VAMP2-eGFP was measured by Western blot with anti-GFP antibody.

Co-immunoprecipitation

We obtained protein samples from medullary TAL suspensions lysed in buffer containing 150 mM NaCl, 50 mM HEPES (*p*H 7.5), 5 mM EDTA, 1% Triton X-100, 0.1% SDS and protease inhibitors. 200 μg of protein from TALs were pre-cleared 30 min at 4ºC with proteinG/proteinA-coupled agarose beads in immunoprecipitation buffer (100 mM NaCl, 50 mM HEPES (*p*H 7.5), 5 mM EDTA, 1% Triton X-100, 1% CHAPS and protease inhibitors). Beads were precipitated by centrifugation and discarded. Supernatants were incubated with 5 μg of rabbit anti-NKCC2 IgG overnight at 4ºC. Control tubes were incubated with a non-immune rabbit IgG. Next day, proteinG/proteinA-coupled agarose beads were added in two sequential rounds for a total of 4 hrs at 4ºC. At the end of the incubation period, beads were sequentially washed in immunoprecipitation buffer, 50 mM HEPES/500 mM NaCl buffer and 50 mM HEPES buffer. Proteins were extracted by incubation at 37ºC in loading buffer and VAMPs were detected by polyacrylamide gel electrophoresis and Western blot. For VAMP2 and VAMP3 immunoprecipitation we used 100 μg of TAL protein lysate and 5 μg of anti-VAMP2, anti-VAMP3 or non-immune rabbit IgG. Beads were eluted as indicated and NKCC2 detected by Western blot.

GST pull down in thick ascending limb lysates

Generation of GST constructs

We generated GST-fusion proteins by sub-cloning the amino-terminus of NKCC2 and a portion of the carboxy-terminus of NKCC2 in GST bacterial expression vectors (named GST-N-NKCC2 and GST-C-NKCC2, respectively). The carboxy-terminal region of NKCC2 was a 71 amino acid- long sequence known to contain an apical localization signal (177). We transformed *E.coli* bacteria with GST, GST-N-NKCC2 or GST-C-NKCC2 and induced expression of the GST fusion proteins by incubating with 0.2 mM IPTG for 4 hours. We lysed the bacteria by sonication in lysis buffer containing 20 mM Tris HCl (*p*H 7.4), 200 mM NaCl and 10 mM EDTA. We purified the GST proteins by incubating with glutathione-coupled sepharose beads in bacterial lysis buffer overnight at 4ºC.

GST pull down

We lysed TAL suspensions from rats to obtain a protein homogenate as described above. We pre-cleared 200 µg of protein sample by incubating with sepharose beads already bound to the GST protein generated before. After one hour of incubation at 4ºC, we centrifuged the samples, eliminated the beads and incubated the supernatants with sepharose beads already bound to GST, GST-N-NKCC2 or GST-C-NKCC2. We incubated overnight at 4ºC. Then we washed with 50 mM HEPES/500 mM NaCl buffer and 50 mM HEPES buffer. We eluted the beads by incubation at 37ºC in Laemmli loading buffer. Samples were run by electrophoresis in 12% polyacrylamide gels and VAMP2 detected by Western blot as described above.

Immunolabeling of surface NKCC2, VAMP2 and VAMP3 in thick ascending limb cells

Cultured TAL cells were transfected with adenoviruses carrying VAMP2-eGFP or VAMP3-eGFP under control of a CMV promoter for 24 hours. The VAMP2- and VAMP3-

eGFP constructs were sub-cloned into the VQ Ad5CMV K-NpA adenovector. Viral particles were produced and purified by Viraquest. Protein trafficking was rapidly stopped by adding cold medium and incubating the cultures at 4°C for 30 minutes. Cells were blocked with 5% albumin in physiological solution for 30 minutes at 4°C. Then the primary rabbit antibody against an exofacial epitope on NKCC2 was added to the apical bath (1/50) at 4ºC for 2 hours. Cells were washed and incubated with the secondary anti-rabbit antibody conjugated with Alexa Fluor 568 (1/100) for 1 hour at 4ºC. After washout, surface VAMP2/ 3-eGFP was labeled with Alexa Fluor 488-conjugated anti-GFP antibody (1/100) for 1 hour at 4ºC. Finally cells were fixed in 4% paraformaldehyde and mounted. Images were acquired using a laser scanning confocal microscopy system (Visitech, Sutherland, England) with a 488 nm diode or Kr/Ar 568 nm gas laser excitation controlled by an acousto-optic tunable filter. Images were acquired at 100x (1.4 NA) and fluorescence observed using 525/55-nm Band-pass or 590-nm Low-pass filters, respectively. Image files (TIFF format) were minimally deconvolved with Autoquant software (Media Cybernetics, MD) using 2-D blind deconvolution. Images from both channels were aligned and pixel-by-pixel co-localization measured using a minimum Mander's overlap coefficient of 0.95. An image for overlapping pixels was generated.

Blood pressure measurements

Systolic arterial pressure was measured by tail cuff in pre-trained mice with MC4000 Multi Channel Blood Pressure Analysis System (Cary, NC).

Measurements of urine parameters

Mice were placed in metabolic cages (Techniplast, Exton, PA) with free access to food and water. After a period of three days of acclimatization, urine samples were collected for 24 hours and volume measured. Then we measured urine osmolality by

freezing point depression with Advanced Model 3300 Micro-Osmometer (Advanced Instruments Inc. Norwood, MA). We also measured urinary Na, K, Cl, and Ca with a StatProfile pHOx Ultra Analyzer (Nova Biomedical, Waltham, MA). Then mice were restricted from access to water for 24 hours. During that period urine was collected to measure the parameters again.

Statistical analysis

Results are expressed as mean ± standard error. One-way analysis of variance (ANOVA) was used to determine differences between means in treatments with more than two groups. Post hoc analysis was performed when differences between means was found using the Bonferroni correction for multiple comparisons. For comparisons between two groups we used Student's *t*-test and paired *t*-test for paired samples. $p<0.05$ was considered significant.

APPENDIX A

List of acronyms

ANOVA: Analysis of Variance

ANP: Atrial natriuretic peptide

ATM kinase: Ataxia telangiectasia mutated kinase

AVP: Arginine-vasopressin

β-AR: Beta-adrenergic receptor

cAMP: 3'-5'-cyclic adenosine monophosphate

cGMP: 3',5'-cyclic guanosine monophosphate

DTT: Dithiothreitol

E. coli: *Escherichia coli*

ENaC: Epithelial Na channel

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFP: Green fluorescent protein

GSK-3: Glycogen synthase kinase-3

GST: Glutathione *S*-transferase

IACUC: Institutional Animal Care and Use Committee

IBMX: 3-Isobutyl-1-methylxanthine

IgG: Immunoglobulin G

IPTG: Isopropyl β-D-1-thiogalactopyranoside

MAL: Myelin and lymphocytes-associated protein

N^6-Bnz-cAMP: N^6-Benzoyl-cAMP

NHE: Na/H exchanger

NHS: *N*-Hydroxysulfosuccinimide

NIH: National Institutes of Health

NKCC2: Na/K/2Cl co-transporter

OSR1: oxidative stress response 1 kinase

PFU: Plaque-forming unit

PKA: Protein kinase A

PKC: Protein kinase C

PTH: Parathyroid hormone

QTL: Quantitative trait loci

ROMK: Renal outer medullary K channel

SCAMP2: Secretory carrier membrane protein 2

shRNA: Short hairpin RNA

siRNA: Small interfering RNA

SNAP: Synaptosome-Associated Protein

SNARE: Soluble NSF Attachment protein Receptors

SPAK: Ste20-related proline-alanine-rich kinase

TAL: Thick ascending limb

VAMP: Vesicle Associated Membrane Protein

VAMP3 -/- : VAMP3 knockout

APPENDIX B

IACUC approval

Henry Ford Health System

Institutional Animal Care and Use Committee
Amendment to Approved Protocol

Please submit one copy containing an original signature to the Research Administration office and email an electronic copy to IACUC@hfhs.org.

PROJECT TITLE: SALT ABSORPTION BY THE TAL: ROLE OF NKCC2 TRAFFICKING

IACUC No.: 1018

PRINCIPAL INVESTIGATOR: PABLO ORTIZ, PH.D.

DEPT: INTERNAL MEDICINE, HYPERTENSION & VASULAR RESEARCH DIVISION **TELEPHONE:** 313-916-7164 **EMAIL:** portiz1@hfhs.org

HOW SHOULD THE APPROVED AMENDMENT BE RETURNED?:
☒ interoffice mail – specify location to mail it to: E&R - 7044
☐ email contact person for pick up from IACUC Office

I am requesting a change regarding the following criteria:

Check all that apply	Complete section(s)
☐ Protocol title[2]	1
☐ Change in Principal Investigator	2
☐ Change in funding source[2]	3
☒ Addition/deletion of research staff[1]	4
☐ Methodology	5
☐ Restraint system	6
☐ Anesthetic/analgesic regimen	7
☐ Postoperative care	8
☐ Euthanasia and second assurance of death	9

Check all that apply	Complete section(s)
☐ Housing requirements[1]	10
☐ Type of caging[1]	11
☐ Cage population density[1]	12
☐ Diet/food, water restriction	13
☐ Increase in animal numbers	14
☐ Location of animal procedures	15
☐ Strain	16
☐ Hazardous agents	17

[1]May be administratively approved by the Veterinarian
[2]May be administratively approved by the IACUC Coordinator

Signature is to be handwritten. Signature stamps are not acceptable.
The information provided in this form accurately represents the changes proposed regarding my previously approved IACUC application. I assure that the certifications that I agreed to in the original application in Attachment 1 will remain in effect.

Signature of Principal Investigator — Date: 03-17-10

IACUC USE ONLY:
Date stamp-received:

RECEIVED
MAR 29 2010
RESEARCH ADMINISTRATION

Signature of IACUC Chair or Designate

IACUC APPROVAL STAMP
APPROVED
APR 06 2010
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

1. **Change in protocol title or additional protocol title:**
   a. **Explain why the change is necessary.**

   b. **New title to replace title stated at the top of this amendment:**

   c. **Additional title requested:**

2. **Change in Principal Investigator (PI)**
   a. **Please explain why there is a change in PI. If the PI is new to HFHS, the PI must meet with the IACUC Coordinator and Bioresources.**

   b. **Name of new PI:**
   c. **Phone:** **Email address:**

3. **Change in funding source**
   a. **Previous funding source:**
   b. **New funding source:**
   c. **If new funding source is NIH, please provide the following:**
      i. **Grant title:**
      ii. **Date submitted to NIH:**

4. **Addition/deletion of research staff**
   a. **Please explain why the change is necessary.**
   New staff person works as graduate student in the project

   b. **Name of new staff person:** Caceres, Paulo
   c. **Person's responsibility:** Terminal surgery (nephrectomy), survival surgery (adenovirus injections), euthanasia
   d. **Is the person enrolled in the Occupational Health and Safety Program?**
   ☒ **Yes**
   ☐ **No, explain why.**

   e. **Complete a *User Qualification* form and submit with the amendment. The User form is on the IACUC website via http://henry.hfhs.org.**

5. **Change in methodology**
   a. **Please describe the proposed change.**

   b. **Explain why the change is necessary.**

   c. **Explain how the change impacts statistical significance.**

   ☐ **Not applicable**

   d. **Will this change the Category of Discomfort classification?**
   ☐ **No** ☐ **Yes (If so, please see Appendix 1)**

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ABSTRACT

REGULATION OF NKCC2 TRAFFICKING BY VESICLE FUSION PROTEINS VAMP2 AND VAMP3 IN THE THICK ASCENDING LIMB

by

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Major: Physiology

Degree: Doctor of Philosophy

The thick ascending limb (TAL) in the kidney regulates extracellular fluid volume and blood pressure. The Na/K/2Cl cotransporter NKCC2 plays a central role in NaCl absorption by the TAL and blood pressure. NKCC2 trafficking to the apical membrane is a major mechanism to control NKCC2 activity. However, little is known about the proteins that mediate NKCC2 trafficking. Inhibition of the vesicle fusion proteins VAMP2 and VAMP3 blunts the increase in surface NKCC2 expression and NaCl absorption in response to stimulation by cAMP. In other cells, VAMPs mediate fusion of exocytic vesicles with the plasma membrane. Whether VAMP2 and VAMP3 mediate different pathways for NKCC2 exocytic delivery to the plasma membrane is not known. We hypothesized that VAMP2 and VAMP3 interact with NKCC2 in the TAL and differentially mediate constitutive and cAMP-stimulated exocytic delivery of NKCC2 to the plasma membrane. We silenced VAMP2 and VAMP3 *in vivo via* shRNAs in rat TALs and measured NKCC2 exocytic delivery and steady-state surface NKCC2 by surface biotinylation before and after cAMP stimulation. We observed that silencing VAMP2 decreased cAMP-stimulated exocytic delivery of NKCC2 and steady-state surface NKCC2 expression, whereas it did not affect

constitutive NKCC2 trafficking in the absence of cAMP stimulation. Silencing VAMP3 decreased total NKCC2 expression and blunted constitutive NKCC2 exocytic delivery and steady-state surface NKCC2 expression, but had no effect on cAMP-stimulated NKCC2 trafficking. We also found by co-immunoprecipitation that VAMP2 and VAMP3 interact with NKCC2 and observed NKCC2 co-localization with VAMP2 and VAMP3 at the apical surface of TAL primary cultures. Interestingly, they co-localized at discrete micro domains at the apical surface. cAMP enhanced NKCC2-VAMP2 interaction, VAMP2 exocytic delivery and co-localization with NKCC2. Finally, to correlate NKCC2 trafficking with a renal phenotype, we studied renal function and blood pressure in VAMP3 knockout mice (VAMP2 knockouts die *in utero*). Urine volume was increased, urine osmolality decreased and Ca excretion was enhanced in VAMP3 knockout mice. This phenotype is similar to NKCC2 knockouts as described previously. When challenged to 24-hours water deprivation, VAMP3 knockouts experienced enhanced Na, Cl and K loss in the urine. When fed a low-salt diet, VAMP3 knockouts excreted excess Na after 24 hours, but then adjusted to normal excretion after 48 hours. Consistent with this renal phenotype, blood pressure was decreased in VAMP3 knockouts. Altogether, this phenotype is consistent with decreased TAL function and is the first phenotype described in VAMP3 knockout mice. We conclude that VAMP2 and VAMP3 interact and co-localize with NKCC2 in the TAL. VAMP2 mediates cAMP-stimulated NKCC2 trafficking while VAMP3 mediates constitutive trafficking. Also, VAMP3 is required for normal NKCC2 expression, renal function and blood pressure.

AUTOBIOGRAPHICAL STATEMENT

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Education

2014: Ph.D. in Physiology: Wayne State University, School of Medicine, Physiology Department, MI, USA.
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Experience

Graduate Research Assistant: Wayne State University, Physiology Dept, Detroit, MI.2008-present
Research Fellow: Hypertension and Vascular Research, Henry Ford Hosp., Detroit, MI,2006-2008.
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Scholarships and Fellowships

1. Predoctoral Fellowship, granted by the American Heart Association, Midwest Affiliate 12PRE12070224. Period July 2012 - June 2013.
2. Predoctoral Fellowship, granted by the American Heart Association, Midwest Affiliate 10PRE3710001. Period July 2010 - June 2012.
3. Interdisciplinary Biomedical Sciences (IBS) Fellowship, granted by the Graduate School, School of Medicine, Wayne State University. Period August 2008 - June 2010.

Awards (selected)

1. 2013 Centennial Award. Department of Physiology, Wayne State University, 06/2013.
2. Research Recognition Award. Epithelial Transport Group, American Physiological Society, 2013.
3. Professional Development Seminar travel award. American Society of Nephrology, 11/2012.
4. Peter K Lauf Student Travel Award. Ohio Physiological Society, 10/2010.
5. Poster competition winner. WSU–U of M. Joint Physiology Symposium, 08/2010.
6. Predoctoral Excellence in Renal Research. Renal Section, American Physiological Society, 2010.
7. Minority Travel Award. NIDDK, American Physiological Society, 04/2010.
8. Oral presentation,first place. XIII Graduate Student Research Day.Wayne State University. 2009.
9. Golden Key International Honour Society. Member by invitation since 2009.
10. Caroline tum Suden/Francis Hellebrandt Professional Opportunity Award, APS, 04/2008.

Peer-reviewed publications

1. **Caceres PS**, Mendez M, Haque MZ, Ortiz PA. VAMP3 mediates trafficking of the co-transporter NKCC2 in thick ascending limbs: Role in renal function and blood pressure. *J Biol Chem.* 2013 *(submitted)*.
2. **Caceres PS**, Mendez M, Ortiz PA. VAMP2 but not VAMP3 mediates cAMP-stimulated trafficking of the renal co-transporter NKCC2 in thick ascending limbs. *J Biol Chem.* 2013 *(In revision).*
3. Haque MZ, **Caceres PS**, Ortiz PA. β-adrenergic receptor stimulation increases surface NKCC2 expression in rat thick ascending limbs in a process inhibited by phosphodiesterase 4. *Am J Physiol.- Renal Physiol.* 2012, 303(9):F1307-14.
4. Ares GR, **Caceres PS**, Ortiz PA. Molecular regulation of NKCC2 in the thick ascending limb. *Am J Physiol.- Renal Physiol.* 2011, 301(6):F1143-59.
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6. **Caceres PS**, Ares GR, Ortiz PA. cAMP stimulates apical exocytosis of the renal Na-K-2Cl co-transporter NKCC2 in the thick ascending limb: Role of PKA.*J Biol Chem.* 2009, 284(37):24965-71.
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