Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Laura I. Galarza-Paez April 14th 2016

The role of mitochondrial calcium uptake in the cortical collecting duct

by

Laura I. Galarza-Paez

Douglas C. Eaton, PhD
Adviser

Department of Biology

Douglas C. Eaton, PhD
Adviser

Jeff M. Sands, MD
Committee Member

Dieter Jaeger, PhD
Committee Member

Tiffany L. Thai, PhD
Committee Member

2016

The role of mitochondrial calcium uptake in the cortical collecting duct

By

Laura I. Galarza-Paez

Douglas C. Eaton, PhD
Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2016

Abstract

The role of mitochondrial calcium uptake in the cortical collecting duct

By Laura I. Galarza-Paez

Principal cells in the renal collecting duct (CCD) regulate total body salt and water. The principal cells respond differently depending on the origin of the signal. The release of ATP into the blood causes an increase in sodium reabsorption via the epithelial sodium channel (ENaC) whereas luminal ATP is inhibitory. Despite opposing effects, both basal and apical ATP increases intracellular calcium ($[Ca^{2+}]_i$). The cell compartmentalizes ($[Ca^{2+}]_i$) in the mitochondria and helps maintain the polarized effect calcium signals. Mitochondria act as dynamic buffers of intracellular calcium in epithelium by helping uptake calcium and help maintain intracellular homeostasis. Mitochondria can regulate local concentrations of $[Ca^{2+}]_I$ in CCD by localizing near the apical and basal membrane to form mitochondrial bands. Mitochondria sequester $[Ca^{2+}]_I$ and prevent Ca^{2+} from diffusing from one pole of the cell to the other; therefore, helping maintain cellular calcium homeostasis. Mitochondria in principal cells form mitochondrial ER associated membranes creating calcium microdomains for the regulation of cellular processes. The primary mechanism for Ca^{2+} uptake across the mitochondrial outer membrane is via the voltage dependent anion channel (VDAC). I hypothesize that when we inhibit the ability of the mitochondria to take up Ca^{2+} we disturb normal renal function and cell polarity. We obtained VDAC1 knock out (KO) mice and predict that inhibiting calcium uptake in CCD would prevent regulation of ENaC and animals would be unable to properly regulate total body salt and water. To test this hypothesis, VDAC1 knockout mice were given a nominally low salt diet, high-salt diet (8%) or high salt diet with daily IP injection of benzamil (ENaC inhibitor) would be physiologically challenged. $VDAC1^{-/-}$ blood pressure, urine concentration and electrolyte

excretion was significantly different than the WT on the same diet. Kidney morphology, blood pressure and other parameters were measured. Overall, our data show that mitochondrial calcium plays an essential role in normal renal function.

The role of mitochondrial calcium uptake in the cortical collecting duct

By

Laura I. Galarza-Paez

Douglas C. Eaton, PhD
Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology
2016

Acknowledgements

First and foremost, I would like to thank my advisor, Dr. Douglas C. Eaton for his help and guidance. I would like to thank my mentor Dr. Tiffany L. Thai for all her help and for being the best part of my undergraduate education. I would also like to thank all of the Eaton Lab members particularly, Otor Al-Khalili, Billie Jeanne Duke and Dr. Heping Ma whose technical and ethical guidance was essential for this project and my growth and a scientist. I would also like to thank Dr. William Craigen at Baylor University for providing the VDAC1 KO mice. I would also like to thank the other members of my committee Drs. Dieter Jaeger and Dr. Jeff Sands. Finally, I would never had made it though the hard work without the support of Dan, my parents Celia and Luis and my sisters Liz and Lili.

Table of Contents

Chapter

List of Abbreviations

AQP2	Aquaporin 2
BP	Blood pressure
$[Ca^{2+}]_I$	Intracellular calcium
CaM	Calmodulin
CCD	Cortical collecting duct
ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
Grp75	glucose-regulated protein 75
HS	High salt
HSB	High salt benzamil
IP3R	Inositol 1,4,5-triphosphate receptor
IM	Inner medulla
NS	no salt
MAM	Mitochondria associated membranes'
MARKS	Myristoylated alanine-rich C kinase substrate
MCU	Mitochondrial calcium uniporter
Mfn2	Mitofusin 2
mpkCCD	Mouse cortical collecting duct
OM	Outer medulla
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
SERCA	Sarcoplasmic Reticulum Calcium Transport ATPase
TEM	Transmission electron microscopy

UTA-1	Urea transporter A-1
VDAC	Voltage-dependent anion channel
WT	Wild type
KO	Knock out

Chapter I
Introduction

Laura I Galarza-Paez wrote the introduction.

A. THE ROLE OF MITOCHONDRIA IN THE REGULATION OF THE EPITHELIAL SODIUM CHANNELS IN CORTICAL COLLECTING DUCT

The distal nephron is the location of the majority of regulated renal salt and water reabsorption. The cortical collecting duct (CCD) principal cells orchestrate fine-tuning of Na^+ and water reabsorption as well as K^+ secretion. Movement of substances from the tubular lumen to the blood can be through the intercellular space between the epithelia (paracellular) or through the cell facilitated by channels and transporters on the apical and basal side of the cells (transcellular). Alteration in the intracellular pathways that regulate the channels can result in alteration in plasma $Na^+$and K^+, blood volume and systemic blood pressure. Principal cells are polarized and respond differently to stimuli depending on the origin of the signal, whether from the tubular lumen (apical) or from the blood (basal). Additionally, they express a different set of proteins on their apical and basal membrane to facilitate reabsorption of water and salt from the tubular fluid. For example, changes in luminal flow causes the release of ATP into the urine where it works though a G protein coupled purinergic receptor, P2Y2, located on the apical membrane to decrease sodium reabsorption. Apical ATP works through a Ca^{2+} dependent mechanism to inhibit the epithelial sodium channel (ENaC) found on the apical membrane of CCD (1,40). The effects of intracellular calcium $[Ca^{2+}]_{I.}$ on ENaC have been characterized and our current understanding is shown in schematic 1 (36,57,38). In contrast, basal ATP stimulates the ligand-activated non-selective cation channel P2X4 and as a result sodium reabsorption is increased by stimulation of ENaC activity (44,45). Despite the opposite effects on ENaC, both apical and basal ATP signal via an increase in $[Ca^{2+}]_I$. The mechanisms

responsible for maintaining the polarity of Ca^{2+} signals in the CCD have not been previously characterized.

Mitochondria are known for their role in metabolism; however, more recently they have been implicated in $[Ca^{2+}]_{i.}$ signaling pathways. Ca^{2+} is a common second messenger that mediates cell contraction, vesicular fusion, apoptosis, as well as an activator of signaling molecules such as protein kinase C (PKC) and calmodulin (CaM) (2,3). Half a century ago several studies characterized the ability of mitochondria to uptake Ca^{2+}, data that is concurrent with the thermodynamic laws of diffusion (4,5,6). Mitochondria have been proposed to play a role in the polarized response of $[Ca^{2+}]_i$ in epithelial tissue like pancreatic acinar cells, airway epithelia as well as neurons by creating Ca^{2+} microdomains (8,46,47). In neurons mitochondria are spatially localized near the plasma membrane of the synapse to aid with large and local increases in $[Ca^{2+}]_{i.}$; which allow for exocytosis of vesicles containing neurotransmitters without disturbing other Ca^{2+} dependent cell functions (64,65). In pancreatic acinar cells, Ca^{2+} signals are confined to the secretory (apical) pole and mitochondrial localize to bands which have been associated with creating Ca^{2+} hotspots and preventing the movement of Ca^{2+} to the basal pole (46,47). It is possible that mitochondria in the CCD principal cells create similar buffering zones that allow for a polarized response to local increases in $[Ca^{2+}]_{i.}$ and are involved in the regulation of ENaC activity.

B. INTRACELLULAR CALCIUM IN THE REGULATION OF WATER AND SALT HOMEOSTASIS.

The kidneys are dynamic organs responsible for waste excretion and maintaining water and electrolyte balance. Blood and plasma are continually filtered within the glomeruli that

begin at each nephron of the kidney. Additionally, the kidneys interact closely with other organ systems including the cardiovascular system. This renal and cardiovascular partnership is particularly important for the maintenance of extracellular fluid volume by regulating water and salt balance. Abnormalities in the regulation of water and salt homeostasis leads to hypertension. Hypertension is a disorder that affects a 29% of American adults. The heterogeneity of the disease is becoming well established. It is known, for example, that alterations in brain, heart, vascular, endocrine, or kidney physiology can cause changes in blood pressure; however, patients are often treated with standard therapies without identifying the underlying cause. This strategy is inherently limited in its success by the heterogeneity of the origin of disease (19,60). One such example is the hypertension in individuals with a rare hypertensive disorder occurs when mitochondrial Ca^{2+} uptake is impaired by a genetic mitochondrial mutation though the underlying causes of the hypertension are unknown (19).

Mitochondria have a well established role as the powerhouse of the cell, but they are also important for maintaining intracellular levels of calcium $[Ca^{2+}]_i$. In the cortical collecting duct, high concentrations of $[Ca^{2+}]_i$ can have a stimulatory or inhibitory effect on ENaC depending on the origin of the signal. ENaC is involved in the reabsorption of sodium ion from the tubular lumen back into the blood in the CCD of the kidney. Despite the importance of calcium in the regulation of ENaC and other water and salt channels not much is known about the importance of mitochondrial calcium uptake in water and salt balance.

The aforementioned Chinese family with maternally inherited hypertension showed a defect in mitochondrial Ca^{2+} uptake that was found to be due to misregulation of the voltage dependent anion channel (VDAC), the relatively non-selective outer mitochondrial membrane

channel that is thought to be the primary mechanism for Ca^{2+}uptake across this membrane (16,19). VDAC is the most abundant protein on the outer membrane of the mitochondria and controls the flux of small molecules including NAD, ATP superoxide and Ca^{2+} (15). VDAC plays a central role in multiple cell processes, including metabolite flux, metabolic compartmentalization and apoptosis (23). There are three isoforms of VDAC (VDAC 1, VDAC2 and VDAC3) which are genomically encoded proteins; therefore, knock out (KO) animals exist for VDAC1 and VDAC3. The three isoforms have similar properties but play significantly different roles in cell function. Silencing VDAC1 causes apoptosis (16,24, 61); while VDAC2 has the opposite effect (16,61). Most of the work done on VDAC isoforms has examined mitochondrial chemistry and the mitochondria's role in physiology aside from apoptosis and cell respiration have not been investigated.

C. THE EXISTENCE OF MITOCHONDRIAL ER ASSOCIATED MEMBRANES AND THEIR ROLE IN THE REGULATION OF ENAC

Calcium is a second messenger that regulates eukaryotic cellular function. Cellular Ca^{2+} signals are regulated by multiple ion channels, pumps and exchangers; the ER and mitochondria play a key role in maintaining cellular Ca^{2+} homeostasis. Intracellular levels of Ca^{2+} are maintained in part by storage in the endoplasmic reticulum (ER). Mitochondria have been observed to localize near ER Ca^{2+} channels like Inositol 1,4,5-triphosphate receptor (IP_3R) (8). The close proximity of mitochondria to the ER allow them to sense cellular Ca^{2+} signals; therefore, acting as highly localized buffers (7,8). This association between the mitochondria and the ER are called 'mitochondria associated ER membranes' (MAM) (7). The close proximity

between the two organelles has been previously observed in multiple tissues (7,8). The physical link between the ER and the mitochondria is facilitated by the molecular chaperone glucose-regulated protein 75 (Grp75) that binds the IP_3R on the ER membrane and VDAC1 on the outer membrane of the mitochondria as shown in schematic 2 (10,11). The protein mitofusin 2(Mfn2) participates in tethering the membranes of both organelles (12). Disruption of Mfn2 in hepatic cells leads to alteration of insulin signaling pathways and glucose homeostasis; however, no research exists on its role in other tissues (12).. MAMs have been proposed to play an integral role in numerous cellular processes including protein sorting, ER stress, lipid synthesis and trafficking, apoptosis, Ca^{2+} handling and signaling among others (9).

Ca^{2+} signals require a rapid release of the Ca^{2+} stored in the ER, to return to baseline the Sarcoplasmic Reticulum Calcium Transport ATPase (SERCA) transports Ca^{2+} from the cytoplasm into the ER (63). Mitochondria aid SERCA with the removal of Ca^{2+} from the cytosol by buffering the release of Ca^{2+} slowly enough as not to exceed the pumping capacity of SERCA. The juxtaposition between ER and mitochondria created by the MAMs create a subcellular microdomain that allows for rapid changes of Ca^{2+} while maintaining cellular homeostasis (12).

The Ca^{2+} released from the IP3R can be transported into the mitochondria through the voltage dependent anion channel (VDAC) found on the outer membrane of the mitochondria, which localizes to the MAM (10,12). The movement of Ca^{2+} through VDAC into the mitochondrial matrix is primarily driven by the large voltage across the inner mitochondrial membrane created by the mitochondrial proton pump (16). Ca^{2+} movement across the inner mitochondrial membrane (IMM) is facilitated by the selective channel mitochondrial Ca^{2+}uniporter (MCU)(16). The association between the ER and mitochondria facilitate the rapid

uptake of Ca^{2+} by the mitochondria to slow its diffusion to other areas of the cell (13,14,15). MAMs have not been previously identified in renal cortical collecting duct, nor has their importance (or lack thereof) in any signaling pathway in this tissue been established.

D. STATEMENT OF PURPOSE

I have tested the hypothesis that mitochondrial calcium uptake is involved in the regulation of intracellular calcium signals responsible for the regulation of water and salt balance in the cortical collecting duct. To this end, I have used a combination of confocal microscopy, molecular biology and animal experiments. These studies provided the first link between mitochondrial calcium uptake and the regulation of sodium and water homeostasis in the cortical collecting duct.

E. SIGNIFICANCE

While mitochondrial Ca^{2+} signaling has been shown to mediate apoptosis, very little data exist showing that mitochondrial Ca^{2+} mediates cell signaling processes, especially in the kidney. This thesis proposes the novel idea that mitochondria might play a role in creating Ca^{2+} microdomains or buffer zones that help with the regulation of channels and transporters in renal epithelia. If a link is found between mitochondrial calcium and ENaC signaling processes, it will pave the way for the establishment of mitochondrial calcium as an important mediator of cell signaling processes.

The possibility of a crosstalk between the ER and the mitochondria could have implications for the understanding of compartmentalization of signaling pathways within

polarized cells. If the ER as the source of Ca^{2+} and the mitochondria as a buffer are interacting to create highly localized microdomains, it will have implications for understanding normal cell and renal function.

Chapter II

Polarized Effect of Intracellular Calcium on the Renal Epithelial Sodium Channel Occurs as a Result of subcellular Calcium Signaling Domains Maintained by Mitochondria

Authors:
Tiffany L. Thai, Ling Yu, Laura Galarza-Paez, Ming Ming Wu, Ho Yin Colin Lam, Hui Fang Bao, Billie Jeanne Duke, Otor Al-Khalili, He-Ping Ma, Bingchen Liu and Douglas C. Eaton

TLT and DCE conceived and coordinated the study and wrote the manuscript. LG, LY, HCL, HFB, BJD, OA, MW and BL provided technical assistance and advice. HM helped troubleshoot experiments. LG, HCL, and TLT analyzed the data.

Abstract

The renal epithelial sodium channel (ENaC) provides regulated sodium transport in the distal nephron. The effects of intracellular calcium ($[Ca^{2+}]_i$) on this channel are only beginning to be elucidated. It appears from previous studies that the $[Ca^{2+}]_i$ increases downstream of ATP administration may have a polarized effect on ENaC where apical application of ATP and subsequent $[Ca^{2+}]_i$ increase has an inhibitory effect on the channel whereas basolateral ATP and $[Ca^{2+}]_i$ have a stimulatory effect. We asked if this polarized effect of ATP is in fact reflective of a polarized effect of increased $[Ca^{2+}]_i$ on ENaC and what underlying mechanism is responsible. We began by performing patch clamp experiments in which ENaC activity was measured during apical or basolateral application of ionomycin to increase $[Ca^{2+}]_i$ near the apical or basolateral membrane, respectively. We found that ENaC does indeed respond to increased $[Ca^{2+}]_i$ in a polarized fashion, with apical increases being inhibitory and basolateral stimulating channel activity. In other epithelial cell types, mitochondria sequester $[Ca^{2+}]_i$, creating $[Ca^{2+}]_i$ signaling microdomains within the cell that are dependent on mitochondrial localization. We found that mitochondria localize in bands just beneath the apical and basolateral membranes in two different cortical collecting duct principal cell lines and in cortical collecting duct principal cells in mouse kidney tissue. We found that inhibiting mitochondrial $[Ca^{2+}]_i$ uptake destroyed the polarized response of ENaC to $[Ca^{2+}]_i$. Overall, our data suggest that ENaC is regulated by $[Ca^{2+}]_i$ in a polarized fashion and that this polarization is maintained by mitochondrial $[Ca^{2+}]_i$ sequestration.

Introduction

The renal epithelial sodium channel (ENaC) is a known contributor to the development of salt-sensitive hypertension, particularly in African Americans (25-27). Its regulation,

therefore, has been the subject of much research over recent years. The distal portion of the renal tubule functions to fine-tune sodium reabsorption to regulate plasma sodium concentration. This is done transcellularly by movement of sodium through ENaC channels on the apical surface of the cell, followed by basolateral movement through the Na^+/K^+ ATPase. The epithelial cells in this segment must express different proteins on the apical vs basolateral surface of the cell to allow for regulated transcellular ion movement. Polarization of the single layer of epithelial cells lining the tubule is essential for the nephron to sense changes in plasma and tubular fluid composition and regulate ENaC and other membrane proteins appropriately (28-30). ENaC must, therefore, be regulated differently by hormones present in the blood vs the tubular fluid.

The role of intracellular calcium ($[Ca^{2+}]_i$) in ENaC regulation is beginning to emerge. P2Y2 receptors are G protein coupled receptors located on the apical membrane in principal cells, where they function to inhibit ENaC by a Ca^{2+}-dependent mechanism (55,56). $[Ca^{2+}]_i$ inhibition of ENaC is, in fact, a well-known phenomenon and several publications by different investigators, have proposed the following model: Gq coupled receptors activate phospholipase C that causes release of Ca^{2+} through IP3 receptors on the endoplasmic reticulum near the apical plasma membrane where ENaC is expressed (33). Following an increase in $[Ca^{2+}]_i$, protein kinase C (PKC) is activated (33) and, via a separate pathway, Ca^{2+} binds to calmodulin. ENaC is usually held in the membrane by phosphatidyl inositol phosphates, particularly PIP2 and PIP3 (34,35). A protein, myristoylated alanine-rich C kinase substrate (MARCKS), normally stabilizes PIP2 to create an anchoring domain for ENaC (36). When MARCKS is bound by calmodulin or phosphorylated by PKC, this causes loss of MARCKS

from the membrane, destabilization of PIP2, and endocytosis of ENaC. $[Ca^{2+}]_i$ can also activate the ubiquitin ligase Nedd4-2, causing ubiquitination and proteosomic degradation of ENaC (37).

Whereas increases in $[Ca^{2+}]_i$ in the cytosol just beneath the apical plasma membrane most likely inhibit ENaC, research from our group and others suggests that increasing $[Ca^{2+}]_i$ in the cytosol very near the basolateral membrane of the cell may stimulate ENaC (38,39) Basal P2X4 receptors stimulate ENaC in a Xenopus distal tubule cell line (38). P2X4 channels are known Ca^{2+} channels and chelating $[Ca^{2+}]_i$ with BAPTA decreased P2X4-induced ENaC stimulation suggesting that increases in $[Ca^{2+}]_i$ are stimulating ENaC when they originate from the basal pole.

$[Ca^{2+}]_i$ spreading must be somehow prevented in epithelia of the distal nephron in order to observe a polarized effect of $[Ca^{2+}]_i$ on ENaC. In pancreatic acinar and airway epithelia, mitochondria can restrict $[Ca^{2+}]_i$ diffusion by sequestering $[Ca^{2+}]_i$ (40,41). We tested the hypothesis that mitochondria in CCD function in a similar manner to create regions of high and low [Ca2+]i within the cell (Ca^{2+} pools) and that these pools allow the same second messenger to affect the same protein (ie ENaC) in opposing ways, depending on the origin (apical vs basal) of the signal. This allows the cell to respond differently to hormone signals that signal via $[Ca^{2+}]$i depending on whether the hormone is sensed in the serosal or luminal compartment.

Methods

Cells. Experiments used either the 2F3 clone of A6 Xenopus distal tubule cells or mouse mpkCCD cells from the cortical collecting duct. The type of cell used in each experiment was decided by ease of use. 2F3 cells are suited for electrophysiology due to their ability to remain at room temperature for extended periods of time, high expression of ENaC, and low

expression of other channels. mpkCCD cells were used for microscopy experiments since they are easy to transfect and easily take up mitochondrial dyes. Cells were grown on permeable supports to confluency at which time cells were polarized and tight junctions were fully developed. For 2F3 cells, media was supplemented with aldosterone (1.5 μM) to increase ENaC activity. Ionomycin when used, was dissolved in <0.05% ethanol. While ethanol can stimulate ENaC, it has no effect at this concentration (42).

Imaging. To visualize mitochondria, cells were loaded with mitotracker red (Life Technologies) for 30 min before visualization. To visualize ER, cells were loaded with ERtracker blue (Life Technologies) for 10 min prior to imaging. To label the membrane, cells were transfected with PLCδ-1 PH-GFP (1.5 μg/well, Addgene) using the Xfect system (Clontech). Lck-CaMP-EGFP was used to visualize changes in $[Ca^{2+}]_i$ near the plasma membrane of the cell (42). To detect changes in mitochondrial Ca^{2+}, cells were cold loaded with Rhodamine 2-AM (Life Technologies) since cold loading has been previously shown to increase specificity of the dye for mitochondrial vs cytosolic Ca^{2+} (43). MpkCCD cells were incubated with Rhod2 for 1.5 hours at 4°C followed by incubation in media without serum for 4 hours at 37°C. Cells were rinsed briefly prior to use. Cells were imaged using an Olympus FV-1000 confocal microscope. To resolve subcellular distribution of fluorescent markers, z-stacks were obtained using sequential optical slices starting at the basal membrane. All images were taken using the same parameter settings.

Sing*le Channel Patch Clamp.* Single channel patch clamp was performed as previously described (42). Briefly, a microelectrode was filled with physiological buffer (96 mM NaCl, 0.8 mM CaCl, 0.8 mM MgCl, 20 mM HEPES, pH 7.4) and lowered to touch the apical or basolateral

membrane of a single cell. Suction was gently applied so that the membrane remained intact and a >1 GΩ seal was formed. All current was recorded at a holding potential of 0 mV. ENaC was identified by its characteristic channel kinetics and current-voltage relationship. ENaC probability of opening (Po) was analyzed using ClampFit software. Empty patches (those with no apparent activity) comprised 30-50% of all patches and were excluded from study.

Data Analysis and Statistics. To quantify the confocal microscopy experiments Image J was used. The number of pixels in a given area was calculated before and after drug addition. Averages were compared using t-test with a $P<0.05$ considered significant.

RESULTS

$[Ca^{2+}]_i$ influences renal ENaC in a polarized fashion.

We sought to test the hypothesis that ENaC is affected differently by changes in $[Ca^{2+}]_i$ depending from which pole of the cell the signal originates. To do this, we performed single channel patch-clamp as previously described (43) and cells were treated with either 5 μM of apical ionomicin (Figure 1A) or 15 μM of basolateral ionomycin (Figure 1B) to increase $[Ca^{2+}]_i$. After 1-3 minutes of apical application of ionomycin we observed a significant decrease in channel activity measured as probability of opening or P_0. We saw that the decrease in ENaC activity was maintained through 4-6 minutes of ionomycin addition. In contrast, basolateral application of ionomycin increased ENaC activity but only after 13-15. In other words, the effect of $[Ca^{2+}]_i$ on ENaC is dependent from the side of the cell in which the increase in $[Ca^{2+}]_i$ occurs. Note that the difference in time course and concentration here is likely due to limited diffusion of agents administered from the basal side of the cell (see discussion). We hypothesize that this polarized effect is the result of two different Ca^{2+}-mediated signaling

pathways present in the same cell type: beneath the apical surface of the cell, Ca^{2+} activates pathways that inhibit ENaC, but beneath the basal surface, Ca^{2+} activates pathways that stimulate ENaC. Dispite the relatively diffusibility of Ca^{2+}, these two pools of Ca^{2+} must not interact in order for polarization to occur. We hypothesize that mitochondrial Ca^{2+} uptake prevents diffusion of $[Ca^{2+}]_i$ within principal cells to allow for these two pathways to remain separated.

Preventing mitochondrial Ca^{2+} transport destroys the polarized effect of $[Ca^{2+}]_i$ on ENaC.

We next tested whether the polarized effects of $[Ca^{2+}]_i$ on ENaC function were dependent on mitochondrial Ca^{2+} uptake. Mitochondrial Ca^{2+} uptake is dependent on movement through the inner mitochondrial membrane, a process facilitated by the mitochondrial calcium uniporter (MCU) (43) Ru360 is a drug that inhibits the MCU and the voltage dependent anion channel, a channel which may facilitate movement across the outer membrane (44,45). We first tested the effect of apical application of Ru360 alone on ENaC function and found it to have an inhibitory effect which persisted over time (Figure 2A). This is not surprising since inhibiting Ca^{2+} uptake by the mitochondria that lie just beneath the apical plasma membrane likely increases apical $[Ca^{2+}]_i$ slightly, inhibiting the channel. We then performed experiments in which Ru360 was first added to the apical surface of the cell to inhibit mitochondrial Ca^{2+} uptake followed by apical (Figure 2B) or basolateral (Figure 2C) addition of ionomycin while ENaC activity was monitored by patch clamp. Whereas figure 1 shows that apical and basolateral ionomycin normally have very different effects on ENaC Po, the shape of the graphs in figure 2B and 2C (the same experiment repeated in the absence of mitochondrial calcium uptake) are the same, suggesting the polarized effect of $[Ca^{2+}]_i$ on ENaC

is dependent on mitochondrial calcium uptake. ATP has been repeatedly shown to inhibit ENaC when applied apically (a process that is dependent on an increase in $[Ca^{2+}]_i$ (48). Figure 2D shows that in the absence of mitochondrial calcium uptake, apical ATP has a stimulatory effect on ENaC. Notice that in each of the panels of Figure 2B-D, the shape of the response is the same. While Ru360 alone has an inhibitory effect on ENaC that persists over time, adding any agent that increases $[Ca^{2+}]_i$ on the apical or basolateral side of the cell has a stimulatory (or null effect that is likely Ru360 inhibition opposed by stimulation) effect on ENaC in the presence of Ru360. In these experiments, we apply Ru360 to the apical surface of the cell. The inability of mitochondria to take up Ca^{2+} would lead to an increase in regional $[Ca^{2+}]_i$. We know from figure 1 that this would inhibit ENaC.

Mitochondria form bands in the distal nephron.

Mitochondrial bands in pancreatic acinar and airway epithelia have been observed by other groups to form barriers to prevent $[Ca^{2+}]_I$ diffusion by sequestering $[Ca^{2+}]_I$ and slowly releasing it (41, 42). We hypothesized that mitochondria may be localized in bands in the cortical collecting duct to allow for $[Ca^{2+}]_i$ polarization and opposing effects on ENaC. We began testing this hypothesisis by using MitoTracker Red to observe mitochondrial localization in cortical collecting duct cells. We used two live cortical collecting duct cell lines: A6 cells from xenopus CCD and mpkCCD cells from mice. The Z axis view of both mpkCCD cells (Figure 3A) and A6 cells (Figure 3B) demonstrates that in fact mitochondria localize in the cytosol very close to each membrane in the cortical collecting duct. To pinpoint the location of mitochondrial bands with respect to plasma membrane (Figure 3C), we used the fluorescent PIP2 indicator PLCδ-1 PH-GFP to label the apical and lateral membranes. We found the apical mitochondrial

bands are located just beneath the plasma membrane. Release of ER Ca^{2+} through IP_3 receptor channels is known to inhibit ENaC. We hypothesized that in order for the Ca^{2+} exiting the ER through the IP_3 receptors to access ENaC and not be taken up by the mitochondria, sections of the ER would have to lie between the apical mitochondrial barrier and the apical plasma membrane. To observe ER localization in relation to mitochondria, we loaded mpkCCD cells with mitotracker red and ERtracker blue to label the mitochondria and ER, respectively (Figure 3D). We found that the ER does pass the mitochondrial barrier in spots in live cells. It is possible that this localization is necessary for Gq receptor inhibition of ENaC since IP3 receptor-mediated Ca^{2+} release would have to occur between the apical membrane where ENaC is localized and the mitochondrial band where Ca^{2+} would be sequestered in order for the majority of the Ca^{2+} to access the channel. Figure 3E shows TEM of a mouse cortical collecting duct principal cell. Note the localization of mitochondria as indicated by black lines highlighting the bands. Location of ~900 mitochondria in >10 different cells is quantified in Figure 3F.

$[Ca^{2+}]_i$ does not spread in cortical collecting duct

In Figure 3, we show that mitochondria in the CCD form bands underneath the apical and basal membranes. This morphology is similar to other epithelia such as airway in which mitochondrial bands slow Ca^{2+} movement across the cell. Since morphology does not necessarily imply function, we tested whether Ca^{2+} can move across live CCD cells from one membrane to the other in Figure 4. To be able to clearly observe Ca^{2+} beneath the cell membrane at the apical and basal poles, we transfected cells with a membrane tethered Ca^{2+} sensor pN1-Lck-CaMP2 (43). This protein contains a membrane tethering domain from Src and a GFP molecule that is fused to calmodulin so that an increase in $[Ca^{2+}]_i$ beneath the membrane

enhances the fluorescence of the GFP molecule. In figure 4A, we applied ionomycin to the apical surface of the cell and found that $[Ca^{2+}]_i$ increased in the apical but not basal region of the cytosol. Conversely, figure 4 B shows that basal ionomycin increases $[Ca^{2+}]_i$ in the basal but not apical portion of the cytosol, suggesting that $[Ca^{2+}]_i$ is compartmentalized in mpkCCD cells. Figure 4C shows that in the absence of mitochondrial Ca^{2+} uptake, $[Ca^{2+}]_i$ compartmentalization is lost and apical application of ionomycin results in an increase in $[Ca^{2+}]_i$ in both the apical and basal portions of the cytosol.

Mitochondrial bands take up Ca^{2+} following $[Ca^{2+}]_i$ increases in CCD.

To test whether mitochondria take up Ca^{2+} following an increase in $[Ca^{2+}]_i$, we cold-loaded mpkCCD cells with the dye Rhod2-AM which is shown to be specific for mitochondrial Ca^{2+} when cells are cold loaded (44) (Figure 5). After apical application of ionomycin, there was an increase in mitochondrial Ca^{2+} in the apical but not basal mitochondrial band. These data show both that mitochondria in CCD cells do take up Ca^{2+} rapidly following an increase in $[Ca^{2+}]_i$ at the apical pole of the cell and also confirm that there is limited Ca^{2+} movement across the cell since there is no Ca^{2+} uptake by the basal band following an apical increase in $[Ca^{2+}]_i$. Figure 5B shows localization of Rhod2AM in mitochondria and Figure 5C shows that Ru360 prevents Ca^{2+} uptake into mitochondria.

DISCUSSION

The data shown herein are the first to directly show that the effect of increasing $[Ca^{2+}]_i$ at the apical vs basolateral membrane produces a different effect on ENaC function in the kidney. Polarization of $[Ca^{2+}]_i$ implies that $[Ca^{2+}]_i$ is compartmentalized in renal epithelia and does not diffuse between compartments. We show that, in a principal cell line, apical

application of the Ca^{2+} ionophore ionomycin inhibits ENaC activity (Figure 1). The effects of increasing apical $[Ca^{2+}]_i$ on renal ENaC function have been well studied. The most well accepted physiological mechanism by which Ca^{2+} inhibits ENaC occurs downstream of purinergic signaling via the P2Y2 receptor on the apical membrane (31). P2Y2 receptors signal via a Gq coupled receptor pathway to induce Ca^{2+} release from ER stores. This release of Ca^{2+} can act via a variety of signaling pathways including PKC activation to inhibit ENaC (49). This is demonstrated physiologically by the fact that mice lacking PKC have overactive ENaC and salt-sensitive hypertension (33,50). ENaC must be tethered to the apical membrane by phosphatidyl inositols PIP_2 or PIP_3. While both PIPs and ENaC are rare, they are recruited together by MARCKS. Binding of calmodulin to MARCKS or phosphorylation by PKC leads to removal of MARCKS and therefore ENaC from the membrane. Ca^{2+} may also inhibit ENaC directly or activate the ubiquitin ligase Nedd4-2 to tag ENaC for proteosomal degradation (37,51).

Interestingly, our data also show for the first time that ENaC is stimulated by basolaterally applied ionomycin (Figure 1B). It is of note that stimulating ENaC requires a very high dose of ionomycin and takes much longer than apical ionomycin to cause an effect. This could imply that effects of basal $[Ca^{2+}]_i$ on ENaC are not physiologically relevantIt is likely that the delay in response and high dose of ionomycin are due to the larger volume of media on the basolateral side of the cells, small size of the pores in the polyester membranes used as a surface in our experiments, and invagination of the basal membrane limit the diffusion of ionomycin so that a larger dose and more time are needed. Unfortunately determining whether this is the case would be difficult to test experimentally.

What is not clear from the present work (or work published previously) are the signaling mechanisms dictating how basolateral increases in $[Ca^{2+}]_i$ work to stimulate ENaC activity. Our previous work shows that basolateral P2X4 receptor stimulation of ENaC is dependent on PI3 kinase (PI3K) and Ca^{2+}. Since Ca^{2+} is not known to modulate PI3K directly, there must be an intermediate signaling molecule mediating the process. Src is one protein that can be regulated by Ca^{2+} and can modulate PI3K and has been shown to regulate ENaC (52). It is possible, therefore, that src is an intermediate in P2X4 signaling or that P2X4 signaling cannot occur without cross-talk from receptor tyrosine kinases upstream of src. Future investigation of these signaling pathways will provide valuable insight into ENaC regulation.

Using two cell culture lines derived from this kidney segment, we observe that in a polarized, live cell culture system, mitochondria form distinct bands at the apical and basal poles of the cells (Figure 3). Bands such as these have been previously identified in other epithelial cell types: pancreatic acinar cells and airway epithelia (40,41). Interestingly, airway and renal epithelia are the primary sites of ENaC expression (53). While the influence of airway mitochondrial bands on ENaC activity has not been addressed, it is interesting that all of the tissues in which banding has been observed rely heavily on ENaC for function. We also show that this banding occurs in vivo since TEM of mouse kidney shows banding in cortical collecting duct principal cells.

In this study, we observed the apical mitochondrial band to be situated just beneath the plasma membrane with portions of the ER jutting through the band. This particular localization is interesting in that it would allow the mitochondria to participate in Ca^{2+} regulation near lipid raft domains. Indeed, ENaC is known to localize in such domains and proteins such as Nedd4-2

and MARCKS, known to regulate ENaC in these domains are influenced by $[Ca^{2+}]_i$ (36,54). Future experiments should be aimed at determining whether disrupting mitochondrial Ca^{2+} uptake influences lipid raft Ca^{2+} signaling.

Using innovative Ca^{2+} sensors for $[Ca^{2+}]_i$ beneath the membrane and mitochondrial Ca^{2+}, we show that $[Ca^{2+}]_i$ is taken up by mitochondria following apical application of ionomycin and that $[Ca^{2+}]_i$ stimulated by ionomycin does not spread throughout the cell (Figure 4). To visualize mitochondrial Ca^{2+}, we used the dye Rhod2-AM (Figure 5). This dye can sense $[Ca^{2+}]_i$ as well but has a preference for mitochondrial Ca^{2+} when cold loaded and seemed to localize in a band in our study (44). We were really only able to visualize the apical mitochondrial band using Rhod2-AM. This could be due to the low uptake of the dye and the faintness of the basal band compared to the apical one. For this reason, a limitation of our study is that we are unable to measure Ca^{2+} uptake by the basal mitochondrial band. We did see the Ca^{2+} in the apical mitochondrial band increase following apical ionomycin application, indicative of Ca^{2+} sequestration. To visualize $[Ca^{2+}]_i$ we used the membrane tethered $[Ca^{2+}]_i$ sensor Lck-CaMP (19). This vector consists of an EGFP molecule fused to calmodulin and the membrane domain of Src. When Ca^{2+} increases at the membrane, the conformation of calmodulin changes such that GFP is excited. This vector helped us to see $[Ca^{2+}]_i$ just under the membrane and determine whether $[Ca^{2+}]_i$ spread following ionomycin application. We found that mitochondria sequester $[Ca^{2+}]_i$ following apical increases in $[Ca^{2+}]_i$ and that when $[Ca^{2+}]_i$ is increased on one side of the cell, it does not migrate to the opposing pole.

Mitochondria transport Ca^{2+} across the inner mitochondrial membrane via the mitochondrial calcium uniporter (MCU)(31). Ru360, a ruthenium compound used in this study

to obliterate the polarized effects of $[Ca^{2+}]_i$ on ENaC, inhibits MCU as well as a component of outer mitochondrial membrane Ca^{2+} uptake (46,56). In the absence of mitochondrial Ca2+ uptake, a similar biphasic response of ENaC to $[Ca^{2+}]_i$ was observed regardless of which pole ionomycin was added to (Figure 2). These data suggest that mitochondrial Ca^{2+} uptake is required for the polarized response of ENaC to $[Ca^{2+}]_i$.

It is interesting to note that mitochondrial Ca2+ uptake has been implicated in blood pressure regulation in a Chinese family that displays maternally inherited hypertension (57). The data presented herein provide groundwork for future investigations into the role of mitochondrial Ca^{2+} uptake in ENaC regulation in salt-sensitive hypertension.

Overall, we conclude that the renal epithelial sodium channel ENaC likely exists in microdomains of Ca^{2+} signaling and that these domains are maintained by belts of mitochondria within the renal epithelial cell. Since this is the first work suggesting the existence of mitochondrial barriers to $[Ca^{2+}]_i$ movement in renal epithelia, much work remains to be done to determine what role these barriers play in the regulation of other proteins or systemic blood pressure.

Chapter III

The Importance of Mitochondrial Calcium Uptake in the Regulation of Renal function

Laura I Galarza-Paez, Douglas C. Eaton, Li Zou, Valerie A. Link, Qiang Yue, Otor Al-Khalili, Billie Jeanne Duke, Heping Ma and Tiffany L. Thai.
LG, DCE and TLT conceived and coordinated the study. LG wrote the manuscript. LG, LZ, BJD, OA, VAL, HM, DCE and TLT and provided technical assistance and advice. LG, TLT and LZ helped troubleshoot experiments. LG and TLT analyzed the data.

The kidneys have a close partnership with the cardiovascular system. Kidneys maintain blood volume, regulate plasma osmolality and secrete hormones that regulate cardiac performance. The distal nephron is the location of the bulk of regulated salt and water reabsorption. The cortical collecting duct (CCD) principal cell in particular, fine-tunes Na^+ and water reabsorption as well as K^+ secretion(25). Alterations in any of the intracellular pathways

regulating these processes can result in alterations in plasma Na+, K+, blood volume, and systemic blood pressure. For these reasons, intracellular signaling in these cells must be closely regulated and precise. In principal cells, Na^+ is reabsorbed from the lumen via the epithelial sodium channel (ENaC), a selectively permeable Na^+ ion channel found solely on the apical membrane of epithelia including principal cells (1). The activity of ENaC is regulated by the numerous intrinsic and extrinsic factors that affect the expression, the activity of each channel and intracellular trafficking.

Mitochondria are essential for cellular metabolism, but they are also important in maintaining intracellular calcium ($[Ca^{2+}]_i$) homeostasis and can contribute to the polarity of calcium signaling in epithelial cells(12,13, 18, 58). Mitochondria can be distributed in specific regions of the cell in close proximity to one another forming bands that buffer calcium and prevent the diffusion of calcium (24). Ca^{2+} like any other molecule follows the laws of diffusion and moves from an area of high concentration to low concentration. The banding of mitochondria prevent Ca^{2+} diffusion; therefore, allowing for the formation subcellular calcium microdomains that allow for variations in Ca^{2+} signaling in different regions of a single cell. This buffering process is important because numerous cellular processes depend on Ca^{2+} including exo- and endocytosis, G protein activity, vesicular trafficking, protein kinase activity as well as the activity of membrane ion channels and transporters (64,65,66). Therefore, mitochondrial Ca^{2+} uptake plays a role in the regulation of channels like the ENaC in principal cells of the CCD (18).

Now that we have clearly demonstrated that mitochondrial Ca^{2+} uptake influences ENaC activity by directly applied Ca^{2+} in cell culture it is important to understand whether altering

mitochondrial calcium uptake would have physiological consequences with regards to water and salt homeostasis. The voltage dependent anion channel (VDAC) is the primary means of Ca^{2+} uptake by the mitochondria OMM, when misregulated VDAC has been linked to early onset maternally inherited hypertension (19). Even though deficits in mitochondrial Ca^{2+} uptake have been observed in a short clinical study, the underlying cause of the hypertension remains unknown (19). Because intracellular calcium has been shown to regulate proteins involved in water and salt balance, we tested the hypothesis that misregulation of mitochondrial calcium uptake would cause deficits in water and salt balance. VDAC has three isoforms VDAC1, VDAC2 and VDAC3; however, only VDAC1 and VDAC3 knock outs can be produced. Using VDAC1 knock out mouse we demonstrated that mitochondrial calcium plays a physiologically relevant role in renal function.

Methods

Animals. Heterozygous VDAC1 mice were obtained from William Craigen at Baylor University and bred in house. All animal experiments were performed on wild type (WT) or VDAC1 double knock out littermate controls ages 2-6 months and approved. All experiments were approved by the Emory University Institutional Care and Use Committee (IACUC). Animals were housed on a 12:12 hour light:dark cycle and given free access to food and water. Animals were fed standard lab chow (0.39% Na^+), nominally salt free diet, 8% salt, or 8% salt with a daily intraperitoneal injection of 1.4mg/kg bw of Benzamil (ENaC inhibitor).

Measurement of blood pressure. Systolic blood pressure was measured by tail cuff (BP-2000, Visitech Systems). Ten measurements were taken consecutively for each mouse and averaged.

An overall average for each treatment group was then obtained. Blood pressure were measured before and after two-week diet treatment.

Characterization of renal function of VDAC1 KO mice. The mice were placed in metabolic cages (Techniplast) for 24 hours of acclimation followed by a 24-hour collection period. Water intake, food, body weight and urine volume measurements were take before treatment and two weeks after treatment. Urine collected was used for osmolality, protein and electrolyte measurements. Urine samples were centrifuged at 15,000 rpm for 15 min to separate any soluble substances before analysis. Urine osmolality was measured using a Wescor vapor pressure osmometer.

Histology. After two-week period of diet, animals were sacrificed and perfused with PBS followed by 4% paraformaldehyde. Kidneys were embedded in Paraplast tissue embedding medium (McCormick, Scientific) and cut to 5μm thick sections. Sections were stained with hematoxylin-eosin(H&E) or Masson trichrome (MT). Images were taken using a light microscope with 60x magnification lens.

Urine electrolytes. Sodium and potassium levels were measure from urine samples taken before and after 2 weeks of treatment. Urine was diluted 1:10 with urine diluent (EasyLyte Medica) and analyzed with EasyLyte analyzer (Medica Corporation's).

Transmission Electron Microscopy. VDAC1 KO mice were anesthetized with a cocktail of ketamine:xylazine and perfused via the abdominal aorta with PBS to remove blood followed by a solution of 4% glutaraldehyde. Kidneys were removed and processed and imaged by the Robert P Apkarian electron microscopy facility.

Western blot analysis. After two weeks, animals were euthanized and kidneys were perfused with standard PBS. Kidneys were dissected into cortex, outer medulla (OM) and inner medulla (IM) as identified by morphology. Protein was measured by detergent-compatible protein assay kit (Bio-Rad), and Western blot analysis was performed with SDS-PAGE using in-house antibodies against AQP2 or UT-A1, as described (20,21,22)

Data analysis and statistics. All values are the mean ± SEM. Western blots densitometry was performed with ImageJ and normalized to the average of the normal chow control group. Glomerular size measurements were taken with ImageJ. Graphs and statistical analysis were created using Prism6 (GraphPad). Statistics were performed as One-way ANOVA and Students t-test. P value of <0.05 were considered significant. Groups were compared to normal chow control and same treatment wild type control. A summary of methods is shown in figure 3.0.

Results

Histology of VDAC1 knock out mouse. In figure 3.1 we used hematoxylin-eosin (H&E) stain to characterize the morphology of VDAC1 knock out mouse under different diet treatments. Additionally, transmission electron microscopy was used to examine cellular morphology of VDAC1 knock out mice fed with normal chow. Arrows indicate areas where the tubules appear thinner than WT control and where glomeruli appear to be atrophied. We concluded that mitochondrial calcium uptake is important for normal nephron morphology.

Accumulation of collagen around the glomerulus and the interstitial space. In figure 3.2, we used Masson's trichrome stain to visualize the formation of small collagen fiber deposits in VDAC1 KO mice. Figure 3.2A shows urine protein of WT and VDAC1 knock out mice before and two weeks after high salt (HS) and high salt benzamil (ENaC inhibitor) (HSB). After HS treatment

VDAC1 KO mice appear to have slightly more collagen accumulated around the glomeruli and the interstitial space with a pathology score of about 5%. Additionally, there was a significantly higher amount of protein found in the urine of animals on either LS or HS diets. Figure 3.2B shows urine protein of WT and VDAC1 KO mice before and after two-week low salt diet (LS) treatment. Not enough urine was collected from VDAC1 KO mice to measure urine protein levels due to severely decreased urine output (Table 3.1). The graph from figure 3.1, depicts the significant difference in size of the Bowman capsule of WT and VDAC1 KO mice after HS diet from the H&E stain. We concluded that under stress like HS diet, animals have an inability to properly regulate mitochondrial calcium uptake and this has detrimental effects of urinary protein since normally protein is not filtered by the kidneys.

Mitochondrial calcium uptake plays a role in the regulation of blood pressure. We used tail-cuff to measure systolic blood pressure of WT and VDAC1 KO before and after two-week treatment. The left panel of figure 3.3 shows systolic blood pressure of mice before and after two-week treatment with HS and HSB. The right panel shows systolic blood pressure of mice fed LS. VDAC1 KO had a significantly different response to their diet than their WT control counter parts. They had lower blood pressures when fed HS diet and significantly higher blood pressures when fed LS than WT controls under the same treatment. We concluded that mitochondrial calcium uptake plays a role in the regulation of systolic blood pressure.

Inability of mitochondria to take up calcium affects sodium urine excretion and urine concentration. Urine electrolytes were measured using Easylyte analyzer. In the left panel of figure 3.3 we quantified the urine Na^+ of WT and VDAC1 KO before and after two-week treatment with HS or HSB. VDAC1 KO mice had significantly lower Na^+ excretion when fed high

salt than their WT controls. The right panel of figure 3.3 shows urine Na^+ of LS treatment, however, the urine Na^+ from both WT and KO mice was below 25mmol/L which is the minimum range detected by the analyzer. We can observe in figure 3.5 that VDAC1 KO mice concentrated their urine more than the WT mice treated with either HSB or LS. We concluded that calcium uptake by the mitochondria is necessary for proper salt and water balance as well as urine concentration.

Mitochondria play a role in proper water and salt balance. When VDAC1 mice were fed HS or HSB they excreted significantly less urine than the WT controls (Table 3.1) and simultaneously had lower water intakes. Additionally, VDAC1 KO mice under HS treatment had significantly higher levels of Aquaporin 2 (AQP2) in the cortex of the collecting duct as shown in Figure 3.4. We concluded that VDAC1 KO mice have misregulation of water and salt homeostasis when under stress.

Discussion

Our previous work demonstrated that mitochondrial calcium uptake and localization plays an important role in Ca^{2+} signaling and the regulation of ENaC. However, to our knowledge the physiological implication of misregulation of calcium uptake have not been previously explored. This study is the first to link mitochondrial calcium uptake to the regulation of water and salt balance as well as normal kidney morphology. VDAC1 KO mice have almost normal physiological parameters when under no stress as summarized in table 3.1. When placed under the stress of high salt or low salt diet kidneys have diffuse and segmental glomerular inflammation, slight tubule thinning and mild interstitial fibrosis. Under stress VDAC1 KO mice concentrated their urine significantly more than the WT control on the same

treatment. It is important to note that inability to properly regulate mitochondrial calcium uptake severely affects the ability to maintain total body salt and water homeostasis. Understanding the physiological effect of mitochondrial calcium uptake in Ca^{2+} signaling might help with the treatment of hypertension as well as kidney disease.

In figure 3.1, we compared representative histology images of WT and VDAC1 KO mice under four different treatments as well as a TEM image of a VDAC1 KO mouse glomeruli fed normal chow. It appears that the VDAC1 KOs when fed HS have a significantly larger bowman capsule than the WT mice in the same diet. Additionally, the tubules in the cortex seem to become thinner when under the stress of the HS and HSB diet. It is possible that these morphological differences are caused by inflammation causing the formation of fibrotic deposits. Figure 3.2 illustrates that VDAC1 KO mice have extra capillary fibrosis surrounding the bowman capsule as well as mild interstitial fibrosis when under HS treatment. Fibrosis is scarring in response to injury and as a result collagen accumulates in the extracellular matrix of tissue. Glomerular damage can explain the higher levels of urine protein (proteinuria) found in the VDAC1 KO mice on HS diet when compared to the WT. VDAC1 has been identified as a key player in mitochondria-mediated apoptosis through Bcl-2 family and cell proliferation (23,24). Therefore, it is possible that when under stress VDAC1 are unable to properly regulate normal cell proliferation in the kidney. Understanding how mitochondrial calcium uptake regulates renal function could help find new treatment options for hypertension, as a predictor of abnormal kidney function or diabetic nephropathy. A future direction is investigating how mitochondrial calcium affects glomerular filtration and if the treatment continues for longer

than two weeks and if the mice would develop chronic kidney disease(CKD) since fibrosis is an indicator of early stages of the disease.

In figure 3.3, we compared systolic blood pressure of WT mice and VDAC1 KO mice under four different treatments. Interestingly the systolic blood pressure (BP) of the VDAC1 KO mice seems to have an opposing response compared to treatment than the WT mice. We found that, unlike the aforementioned hypertensive Chinese family with decreased VDAC function and hypertension, the VDAC1 KO mouse's blood pressure is not altered under normal chow and is in fact lowered by a high salt diet (19). It is possible that the VDAC1 KO are not responding to the diet since on HS the BP was lower than the WT and on LS the BP was higher as summarized in Table 3.2. Littermate WT controls, however, showed protection against high salt diet. When fed low salt the VDAC1 mice had no significant change in systolic BP when compared to normal chow control, but significantly higher blood pressure than their WT counterparts as summarized in table 3.2. The changes in blood pressure mimic the fibrotic phenotype, where HS VDAC1 KO mice had mild fibrosis. One interesting idea is that VDAC1 mice might have issues with the regulation of peripheral vascular resistance and as a result of hypoperfusion there is inflammation in the kidneys; however, the underlying causes of the changes in BP need to be further investigated.

VDAC1 KO mice have problems with handling water and salt balance. When fed HS they excrete significantly lower amounts of Na^+ than the WT as shown in figure 3.4, while simultaneously excreting lower urine volume (table 3.2). Furthermore, they had a higher abundance of aquaporin (AQP) 2 in the cortex as summarized in figure 3.6. AQP2 is solely found on the apical surface of kidney collecting duct principal cells, and it is responsible for

reabsorption of water from the urine back into the blood (67). Therefore, upregulation of AQP2 in the VDAC1 KO mice could explain the lower urine output volume vs WT controls. On LS diet VDAC1 KO mice seem to concentrate their urine more and have no significant difference in water intake or urine output than the WT mice. It is possible that in response to stress, osmoreceptors in the hypothalamus release the antidiuretic hormone vasopressin causing up regulation of the expression of AQP2 as well as affecting the peripheral vascular resistance that might be causing inflammation to the kidney. UTA1 is also regulated by ADH; however, its expression was down regulated suggesting that the regulatory pathways for both AQP2 and UTA1 are disturbed in VDAC1 KO mice. Since VDAC1 is involved in multiple pathways including Ca^{2+} signaling, exo- and endocytosis, vesicular trafficking and protein production among others; multiple pathways are affected causing changes in normal water and salt channels and transporters (23,64,65,66). Additionally, differences in the expression of both AQP2 and UTA1 in VDAC1 KO mice might be implicated with problems with trafficking.

This work has direct impact on public health by identifying pathways affected by polymorphisms associated with genomic and mitochondrial DNA mutations in hypertensive patients. Western cuisine is often high in salt content; therefore, it is possible that the underlying cause of some unknown high blood pressure cases and kidney damage might be due to mitochondrial maladies. Overall, this study is the first to identify mitochondrial calcium uptake to be an integral part in the regulation of normal water and salt balance. Understanding the underlying cause of the fibrosis, hyponatremia and hypotension can help with the development of treatments for hypertension and kidney disease.

Chapter IV

Mitochondria Associated ER Membranes are Present in Cortical Collecting Duct

Laura I Galarza-Paez, Douglas C. Eaton, Li Zou, Valerie A. Link, , Otor Al-Khalili, Billie Jeanne Duke, Heping Ma and Tiffany L. Thai.
LG, DCE and TLT conceived and coordinated the study. LG wrote the manuscript. LG, LZ, BJD, OA, VAL, HM, DCE and TLT and provided technical assistance and advice. LG, TLT and LZ helped troubleshoot experiments. LG and TLT analyzed the data.

The appearance of mitochondria in eukaryotic cells is thought to have occurred millions of years ago and represents an important step in the evolution of the eukaryotic cell. Indeed, mitochondria serve many cellular functions including, of course, cell respiration. In addition to being the "powerhouse of the cell," mitochondria play many important roles including regulation of intracellular calcium ($[Ca^{2+}]_I$). $[Ca^{2+}]_i$ is an important signaling molecule in all cells. $[Ca^{2+}]_i$ regulates cellular contraction, apoptosis, signaling, vesicular fusion, and many other processes(64,65,66). To ensure that each of these processes remains in balance, the cell must: 1) keep $[Ca^{2+}]_I$ low under basal conditions so that no process is inadvertently activated and 2) compartmentalize $[Ca^{2+}]_I$ within the cell so that signaling processes can be confined to a certain region of the cell. Mitochondria can participate in both of these processes with an emphasis on the latter (8,18,46,47)

It is a well-known fact that in neurons and pancreatic acinar cells, mitochondria can localize to particular regions of the cell to form barriers to $[Ca^{2+}]_I$ diffusion(8,18,46,47). This is established by a process analogous to soaking up a pool of water with a wall of sponges. The mitochondria suck up excessive calcium but, like sponges, have limited capacity and eventually must slowly extrude their built up Ca^{2+} so as to avoid triggering apoptosis, a process that occurs when mitochondrial Ca^{2+} concentrations become too high (9). Thus, the localization of mitochondria in a cell can be seen as walls to $[Ca^{2+}]_I$ movement within the cell.

$[Ca^{2+}]_I$ increases that occur downstream of signaling pathways use two primary routes: entry from the extracellular milieu where Ca^{2+} concentrations are much higher(67) or efflux from Ca^{2+} stores in the sarco/endoplasmic reticulum that are maintained at high levels by activity of the sarco/endoplasmic reticular Ca^{2+} ATPase (8). In addition to their general $[Ca^{2+}]_I$

uptake function, mitochondria have been shown to intimately associate with ER at regions known as mitochondria associated ER membranes (10,11,14). In these regions, a complex of proteins link the two organelles together and, indeed, mitochondria localized near ER show higher concentrations of intra-mitochondrial calcium than mitochondria that are not associated with ER (10,11,14). Extrusion of Ca^{2+} from the ER is mediated by either of two Ca^{2+} channels: the inositol trisphosphate (IP3) receptor or the ryanodine receptor. One particular IP3R subtype (III) associates with the voltage dependent anion channel (VDAC) 1 on the surface of the outer mitochondrial membrane (9). This interaction is both direct and mediated by two other proteins: mitofusin 2 and GRP75 which hold the entire complex together (9,10). Despite its name, VDAC1 is a rather non-selective channel and can transport anions and cations and also small molecules such as ATP. The family of VDAC channels serve as the primary mechanism of Ca^{2+} transport across the outer mitochondrial membrane (16,45,57).

In a recent set of experiments, we investigated the role of mitochondria in calcium uptake in renal cortical CCD principal cells and in the subsequent regulation of the renal epithelial sodium channel (ENaC) (18). In the present work, we tested the hypothesis that mitochondria associate with endoplasmic reticulum in these cells. Using electron microscopy and immunofluorescence on isolated cells and kidneys, we show that these two organelles do, indeed, interact. Since ENaC is regulated by both mitochondrial and endoplasmic reticular calcium pathways, this work may have important implications for the regulation of this protein.

Methods

Animals. Animals were housed and used as described in chapter 3.

Live Cell Staining. MpkCCD cells were grown to confluency on 24 mm transwell inserts and stained with mitotrackerRed and ERtracker blue according to the protocols contained in the package insert immediately prior to visualization on an Olympus FV1000 upright microscope. Z stacks were performed with twenty 1µm thick consecutive images.

Transmission Electron Microscopy. Mice were anesthetized with a cocktail of ketamine:xylazine and perfused via the abdominal aorta with PBS to remove blood followed by a solution of 4% glutaraldehyde. Kidneys were removed and processed and imaged by the Robert P Apkarian electron microscopy facility.

Immunofluorescence. In the cell experiments, cells were washed with PBS followed by a solution of 4% paraformaldehyde. Cells were mounted on a glass slide for staining. For animal experiments, mice were perfused via the abdominal aorta with PBS followed by 4% paraformaldehyde. Kidneys were removed and embedded in paraffin wax, sectioned to 4 µm sections, and mounted on slides. Slides were stained for immunofluorescence by the method used by the Sands group at Emory University (69). VDAC1 was stained with a donkey anti-rabbit polyclonal antibody from Santa Cruz and IP3RIII was stained with a donkey anti-goat polyclonal antibody from Proteintech. Alexa fluor 488 rabbit and Alexa Fluor 568 goat were then used to visualize the proteins. Slides were imaged on an Olympus FV1000 upright microscope.

Data Analysis and Statistics. To determine colocalization, the algorithms of ImageJ coloc2 were used. These algorithms examine merged images pixel by pixel for red and green and provide estimates of colocalization represented in the scatterplots/2D histograms. Pearson's correlation coefficient was used to determine degree of colocalization (17).

Results

Mitochondria and ER lie in close proximity in mouse CCD principal cells. In figure 4.1, we used transmission electron microscopy to observe the localization of ER and mitochondria in CCD principal cells from wild type mice (A) or mice lacking VDAC1 (B). Principal cells were identified by the presence of primary cilia. Black circles show points of contact of the ER and mitochondria. We conclude that mitochondria and ER lie in close proximity in principal cells from both the wild type and the VDAC1 knockout mice.

VDAC1 and IP3RIII co-localize in CCD principal cells both in animals and in cell culture. In mitochondria associated ER membranes, VDAC1 and IP3RIII proteins directly associate. We used immunofluorescence experiments with VDAC1 (green) and IP3RIII (red) antibodies to determine whether these two proteins associate in principal cells. Figure 4.2A shows an image of the cortex of a wild type mouse stained with these two antibodies. The lower panel shows co-localization of the two proteins in the cortex overall and in what we believe to be the CCD. Figure 4.2B shows our method for identifying the CCD. In this image, we again used the VDAC1 antibody (green) but counterstained the principal cells using an antibody against aquaporin 2, a protein known only to be expressed in principal cells. Interestingly, the cells that stained with aquaporin 2 expressed VDAC1 primarily at or near the plasma membrane. We used this unique feature of the CCD to identify CCD as tubules expressing VDAC1 at or near the plasma membrane in panel 4.2A. In 2C and D, we repeated the co-localization experiment in A6 and mpkCCD cells, a frog and mouse principal cell line, respectively. The lower panels show the extent of co-localization of the two proteins in each figure. We conclude from the very high

degree of co-localization of these two proteins in both animals and cell lines that mitochondria and ER directly associate in CCD principal cells.

Discussion

The data outlined herein clearly demonstrate a close association between mitochondria and endoplasmic reticulum in renal cortical collecting duct principal cells. Mitochondria and ER associate in many different tissues but, to our knowledge, this association has never been previously demonstrated for any kidney cell type and these data therefore pave the way for future experiments determining how this association impacts renal function. Our previous work was the first to show that mitochondrial calcium uptake can regulate the activity of ENaC in CCD principal cells(18). One obvious future direction is to determine the role, if any, that association between ER and mitochondria affects this channel. With such a wide array of channels and transporters in the kidney tubules being dependent on $[Ca2+]_i$, the future directions of how MAMs regulate renal channels and transporters are seemingly endless. The Han family in China, a well-studied family with maternally inherited salt-sensitive hypertension, has a gain of function of VDAC activity, though the molecular mechanism underlying this gain of function is unknown (19). Understanding how VDAC and MAMs in general can regulate sodium channels such as ENaC and others in the kidney is therefore a worthwhile endeavor.

In figure 4.1, we compared TEM images of principal cells from wild type and VDAC1 knockout mice. The goal of this experiment was to determine whether mitochondria and ER can associate without VDAC1. In the image of the VDAC1 knockout kidney, the mitochondria and ER still appear to lie in close proximity to one another. One limitation of this study, however, is that we have no way of knowing if the mitochondria and ER directly interact of are

simply in close proximity. Our Immuno fluorescence tells us that they do interact in wild type mice, but since the association is typically via the VDAC1 protein and the VDAC1 antibody has nothing to detect in VDAC1 KO mice, it is impossible to tell using this technique whether or not the two organelles are directly interacting. One interesting possibility is that VDAC2 or 3 are compensating for VDAC1 in the knockout and that MAMs still form. A future direction would therefore be to test for co-localization of each of the other VDAC proteins with the IP3RIII. It is equally plausible, however, that the appearance of ER and mitochondria touching in the EM pictures is misleading. The mitochondria of VDAC1 KO mice appear enlarged, possibly due to altered fission and fusion rates that are known to be a factor in these animals as well as possible compensation for decreased respiration known to be present in these animals by producing more mitochondria (70). Nonetheless, this increased number of mitochondria take up a much larger percentage of the cell and therefore the probability of mitochondria lying near the ER is inherently more likely.

In figure 4.2, we showed co-localization of VDAC1 and IP3RIII in cultured CCD principal cells as well as cortex from a wild type mouse. These data suggest a direct interaction of these two proteins and therefore an interaction of mitochondria and ER in these cells. Interestingly, in the proximal tubules in the mouse cortex (identified by their brush border), we saw almost complete co-localization of VDAC1 and IP3RIII (ie they are only expressed in MAMs). In the CCD while there was still very strong co-localization (around 75%), there was a significant fraction of VDAC1 that localized at or near the plasma membrane and did not co-localize with IP3RIII indicating it is not in a MAM. VDAC1 has been shown to be expressed at the plasma membrane in many other cell types where it can transport Ca^{2+}, but has also been shown to transport

other ions and molecules such as ATP (71). Principal cells of the CCD rely on extracellular ATP to regulate ENaC activity (34,48). Previously this transport was known to be mediated, at least in part, by the activity of connexin hemichannels. Future experiments should be aimed at determining whether plasma membrane VDAC1 also mediates ATP release and whether or not this ATP release determines channel function (72).

Overall, these studies impact the fields of renal physiology and nephrology by showing, for the first time, the association of mitochondria and ER in CCD principal cells. These cell biology experiments lay the groundwork for future studies determine the impact of this interaction on kidney physiology, salt and water transport, blood pressure regulation, and urine concentration.

Chapter V

Discussion

Laura I. Galarza-Paez wrote the discussion.

Overall, this work represents a collective study of mitochondrial calcium uptake in the cortical collecting duct principal cells. In chapter 2 we found that mitochondria localize near the apical and basal pole in bands capable of sequestering calcium to help maintain the polarity of the cell. The work outlined was the first to link mitochondrial Ca^{2+} uptake to the regulation of ENaC. In chapter 2 we investigated the physiological impairments caused by misregulation of mitochondrial Ca^{2+} uptake. We found that under normal conditions, VDAC1 KO have relatively normal renal and cardiovascular function. However, when under the stress of a high salt diet mice developed fibrosis, had problems with urine concentration and sodium excretion. Finally, in chapter 3 we investigated the presence of and mitochondria associated membranes with the ER to form calcium microdomains both in vitro and in vivo. These data have implications to the field of molecular biology, biochemistry, transport regulation, renal physiology, hypertension and disease.

Mitochondrial bands acting as buffers have been previously found solely in pancreatic acinar cells, airway epithelia and neurons. The work presented herein shows that other epithelial cells have these bands and they might not be as “specialized” as once previously thought (40,41). The data highlights the importance of mitochondrial Ca^{2+} uptake in the regulation of ion channels. The differences in expression of proteins like AQP2 and UTA1 in the VDAC1 KO mice when compared to the WT, and appearance of vesicles in VDAC1 KO mice suggest that vesicular trafficking might be affected by disturbing mitochondrial Ca^{2+} uptake in these animals. These experiments may pave the way for future experiments in the role of mitochondria in trafficking.

Aside from mitochondrial Ca^{2+} regulating Cl^- channels in the lung no other experiments have shown mitochondrial Ca^{2+} regulating any channels or transporters anywhere else (41). These experiments support the hypothesis that mitochondrial Ca^{2+} regulates channels in the kidney. Therefore, these results have great implications for transport regulation. The compilation of this work and the results by the Tinel et al. suggests that mitochondria in other epithelial might regulate channels and transporters. This has implications for all channel and transporter diseases like hypertension, urine concentration, acid base balance, cystic fibrosis, asthma and gut reabsorption diseases so they should be studied accordingly.

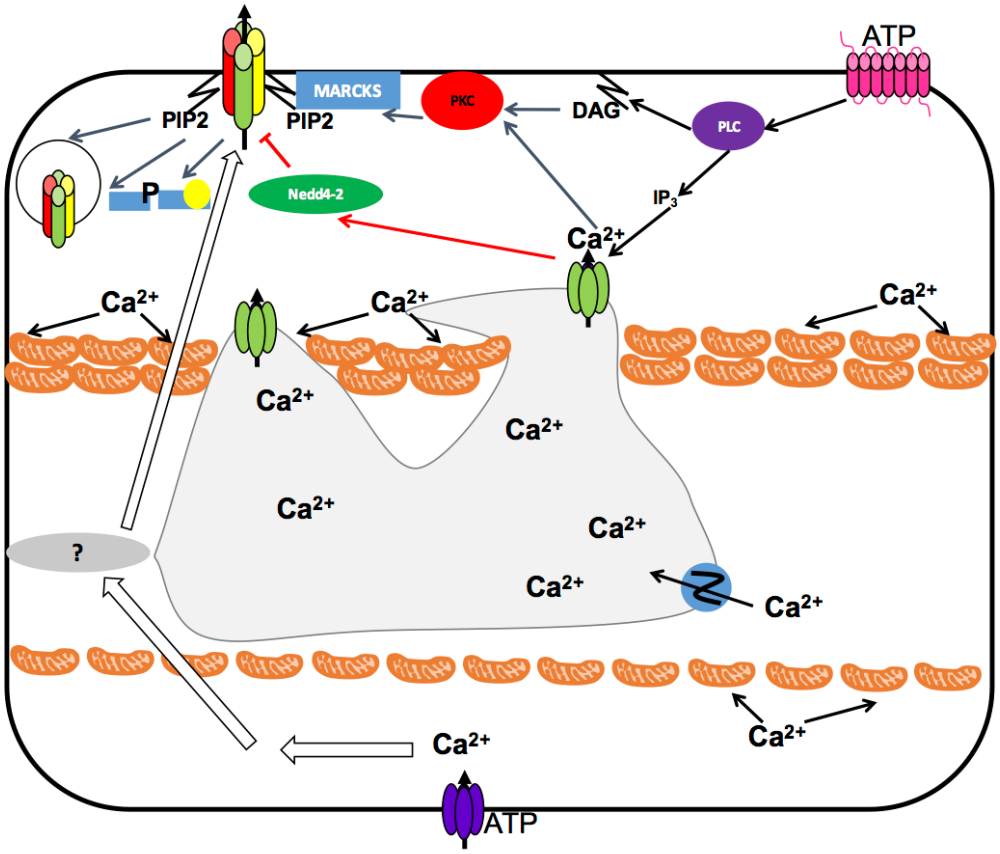
The novelty of these data is that they are the first to implicate Ca^{2+} uptake in the regulation of ENaC in principal cells, as well as the physiological relevance of proper Ca^{2+} buffering in normal renal function. When the ability of mitochondria to take up Ca^{2+} is deceased as is the case in the VDAC1 KO mice, they develop mild interstitial fibrosis, misregulation of sodium and water transport and alteration in urine concentration when physiologically challenged by their diet, indicative of defects in normal water and salt balance in the kidneys. Fibrosis has implications for diabetes, because renal fibrosis is an important issue in diabetic nephropathy. Therefore, this work could help with the development of new treatments by improving our understanding of hypertension and disease.

These data oppose the hypertensive phenotype seen in the VDAC mutation in Chinese humans (19) and suggest that VDAC1 has a detrimental effect on ENaC regulation and blood pressure under altered salt diet conditions rather than a helpful one. Suggesting, that inability to buffer and take up Ca^{2+} has detrimental effects on normal renal function. The findings on the mild fibrosis have implications in the treatment of kidney damage because fibrosis is often one

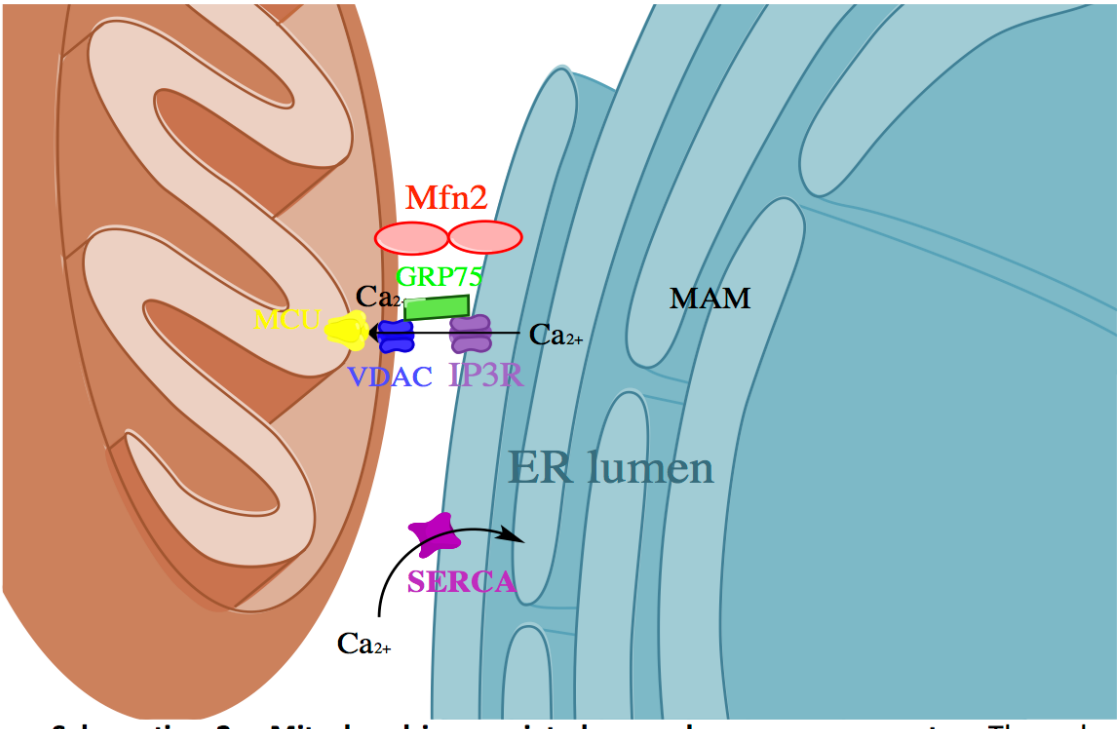
of the first symptoms (73). Furthermore, the damage to the glomeruli can be due to hypoperfusion mimicking the effects of nephropathy (damage to the kidney). VDAC1 was found to localize on the cell membrane of principal cells; however, its function on cell membrane remains unknown. It is possible that membrane bound VDAC1 is involved in mediating ATP release into the tubular lumen, which then activates purinergic receptors causing an increase in $[Ca^{2+}]_I$ that activates a signaling cascade inhibiting ENaC. VDAC1 on the plasma membrane possibly affects the regulation of blood pressure and other disease.

In summary, the data presented in my thesis lays the ground work for future research in the role of mitochondria associated membranes in the principal cells, as well as their functionality or lack of thereof when VDAC1 is silenced. We are one of the first groups to suggest that basolateral increases in $[Ca^{2+}]_I$ stimulate ENaC, but the details of the mechanism remain unknown. It is possible that VDAC3 compensates for some of the VDAC1 roles making it imperative to investigate and characterize renal function in VDAC3 KO mice. Even though plasma membrane VDAC1 has been observed in multiple tissues its role in cell function is not thoroughly understood. A future aim should be to investigate whether VDAC1 channels on the plasma membrane of the cortical collecting duct principal cells act similarly to connexin hemichannels and transport ATP into the cell and whether or not this ATP release determines channel function. Additionally, we could breed a VDAC1/VDAC3 KO mice to eliminate the possible compensatory mechanism. Such experiments should help determine the contribution of mitochondrial calcium in the regulation of signaling pathways to hypertension, diabetes and kidney disease and may help pave the way for the discovery of novel therapies.

Figures



Schematic 1. Proposed Schema of ENaC regulation by intracellular Ca^{2+}. Apical stimulation with ATP works though G coupled purinergic receptor P2Y2. Ca^{2+} released from IP3R inhibits ENaC by decreasing membreane instertion, increasing ubiquitination and decreasing probability of opening. Basal increases in intracellular Ca^{2+} stimulate ENaC by an unknown pathway.



Schematic 2. Mitochondria-associated membrane components. The close interaction between the mitochondria and the ER is essential for rapid uptake of Ca^{2+} by the mitochondria. Ca^{2+} is released from IP3R on the lumen of the ER, the voltage dependent anion channel (VDAC1) found on the outer mitochondrial membrane (OMM) rapidly uptakes the Ca^{2+}. Mitofusin2 (Mfn2) is involved in tethering both organelles, while GRP75 helps facilitate the interaction between IP3R and VDAC.

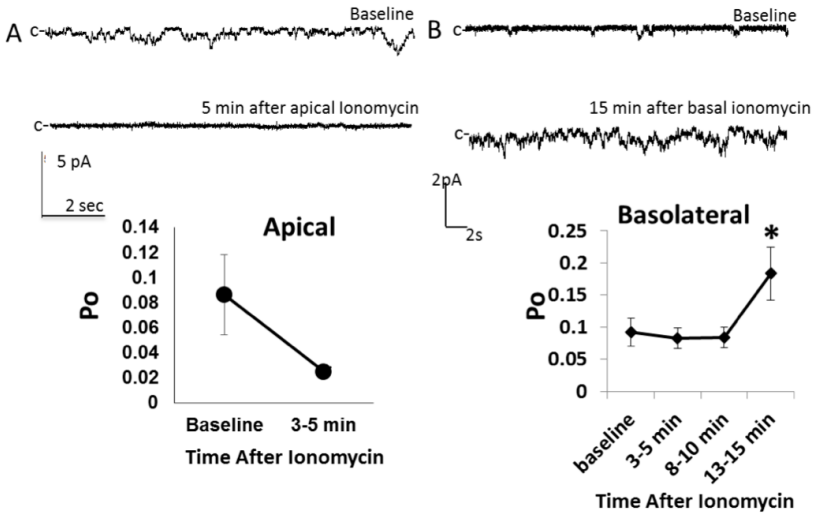


Figure 1: Polarized effects of ionomycin on ENaC activity. Xenopus cortical collecting duct (A6) cells were subjected to single channel patch clamp and treated with apical (A) or basolateral (B) ionomycin (5 or 15 μM, respectively). N>5 per group, *P<0.05.

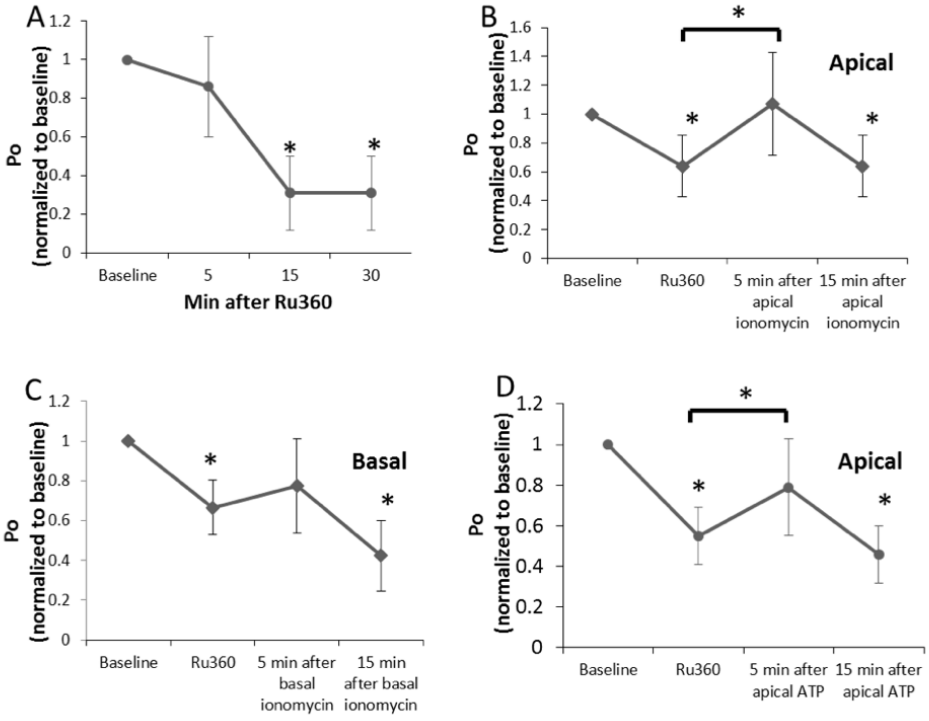


Figure 2: Effect of inhibiting mitochondrial Ca2+ uptake on ENaC activity. A6 cells were subjected to single channel patch clamp then treated apically with 1μM of the MCU inhibitor Ru360. A) shows the effect of Ru360 alone. In subsequent experiments, a second agent was added 5-7 min following apical Ru360: B) Ionomycin was added apically, C) Ionomycin was added basolaterally, and D) ATP was added apically. N≥6 recordings per group, *P<0.05 versus baseline or vs Ru360 (when shown as a bar).

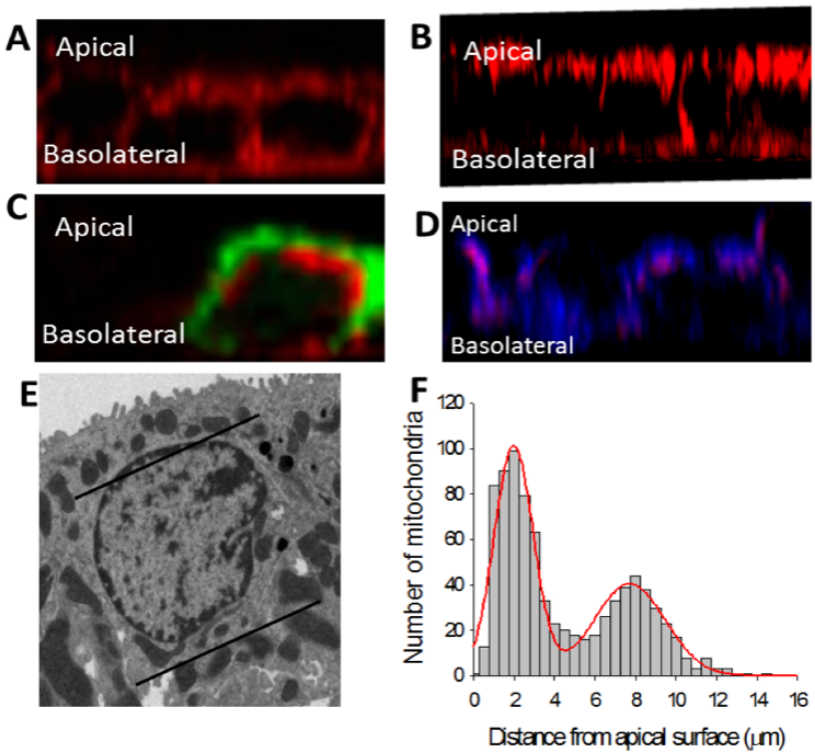


Figure 3: Location of mitochondrial bands in CCD cells. A) mpkCCD cells stained with mitotrackerRed. B) A6 cells stained with mitotrackerRed. C) Localization of mitochondria (red) in relation to plasma membrane (green) in mpkCCD cells. D) Localization of mitochondria (red) with relation to ER (blue) in mpkCCD cells. E) TEM of a principal cell from a mouse kidney. F) Distance of 900 mitochondria from the apical membrane in mouse principal cells Red line is best least-squares fit of the distances to two Gaussian distributions.

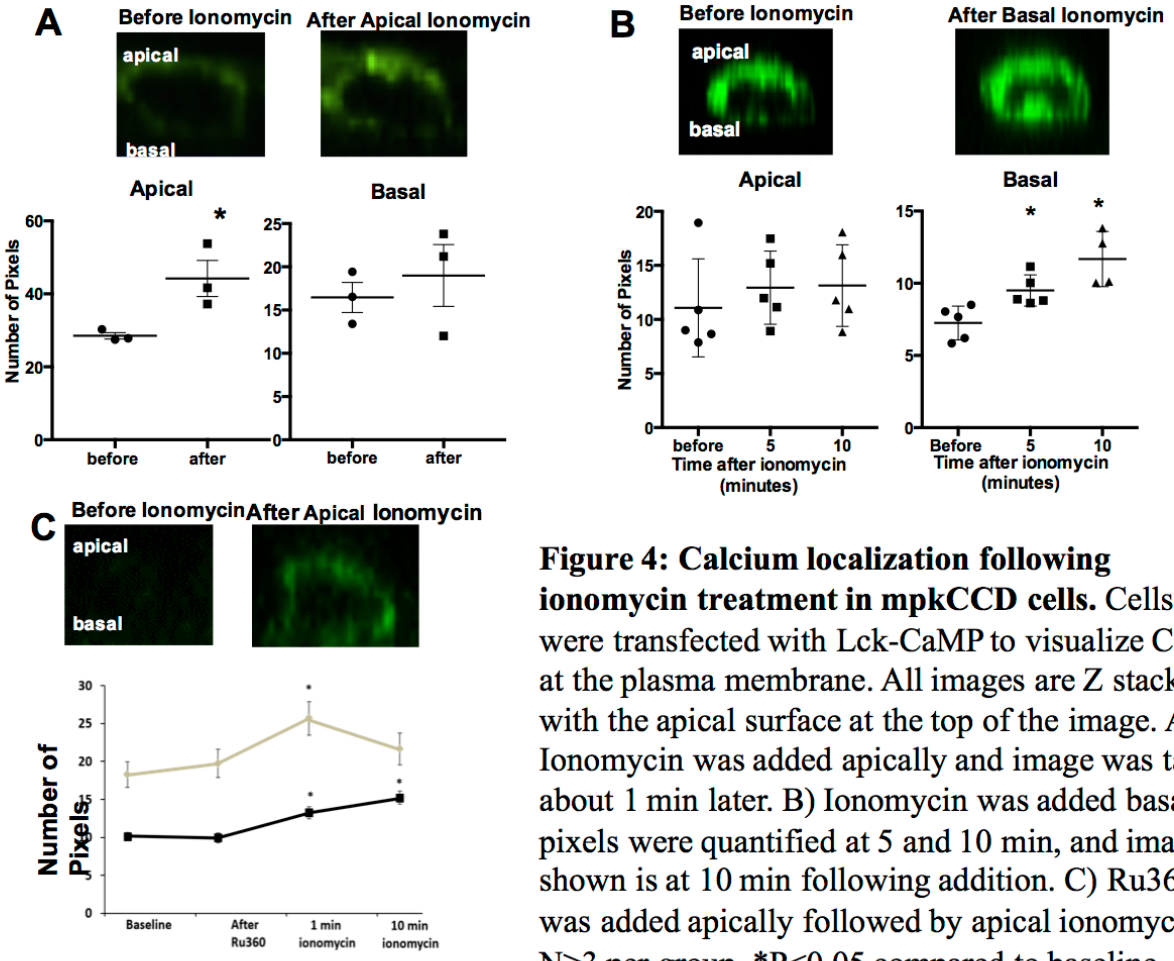


Figure 4: Calcium localization following ionomycin treatment in mpkCCD cells. Cells were transfected with Lck-CaMP to visualize Ca2+ at the plasma membrane. All images are Z stacks with the apical surface at the top of the image. A) Ionomycin was added apically and image was taken about 1 min later. B) Ionomycin was added basally, pixels were quantified at 5 and 10 min, and image shown is at 10 min following addition. C) Ru360 was added apically followed by apical ionomycin. N≥3 per group, *P<0.05 compared to baseline.

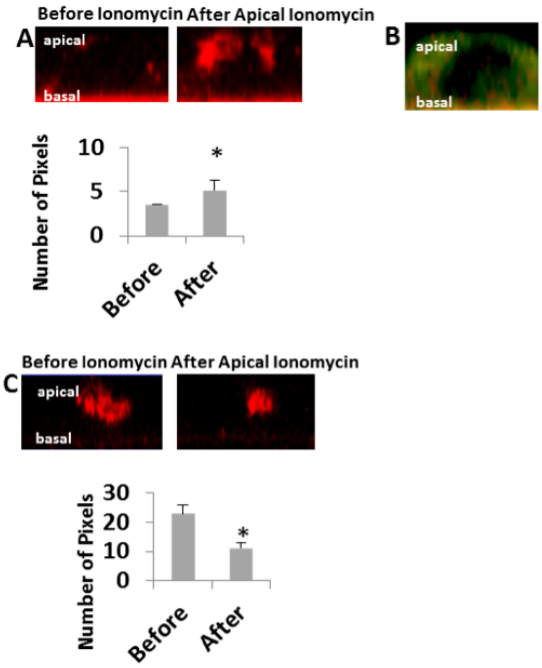


Figure 5: Mitochondrial calcium before and after apical application of ionomycin. mpkCCD cells were stained with Rhod2-AM to visualize mitochondrial calcium. A) Z stacks were obtained before (left panel) and after (right) apical treatment with 5 μM ionomycin. Apical changes in calcium were quantified before and 30s-1 min after treatment. B) Cells were co-stained with mitotracker Green and Rhod2-AM. C) Cells were stained with Rhod2-AM and treated with Ru360 for 20 min prior to apical addition of ionomycin. . Apical changes in calcium were quantified before and 30s-1 min after ionomycin treatment. N≥3 per group, *P<0.05 vs control.

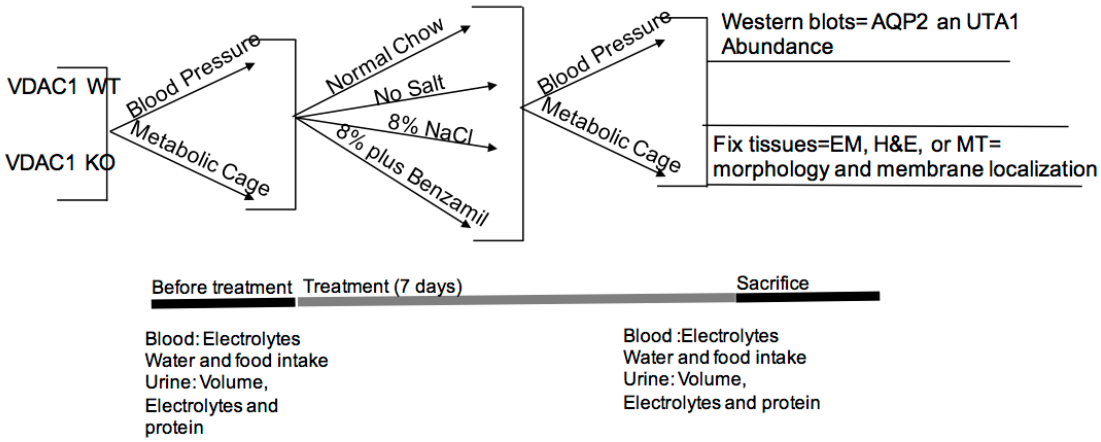


Figure 3.0. VDAC1 methods of characterization of renal function. VDAC1 and WT mice were placed in metabolic cages and their systolic blood pressures were measured before and after 2 week treatment. Mice were euthanized and kidneys were used for EM, H&E, MT and Western blotting.

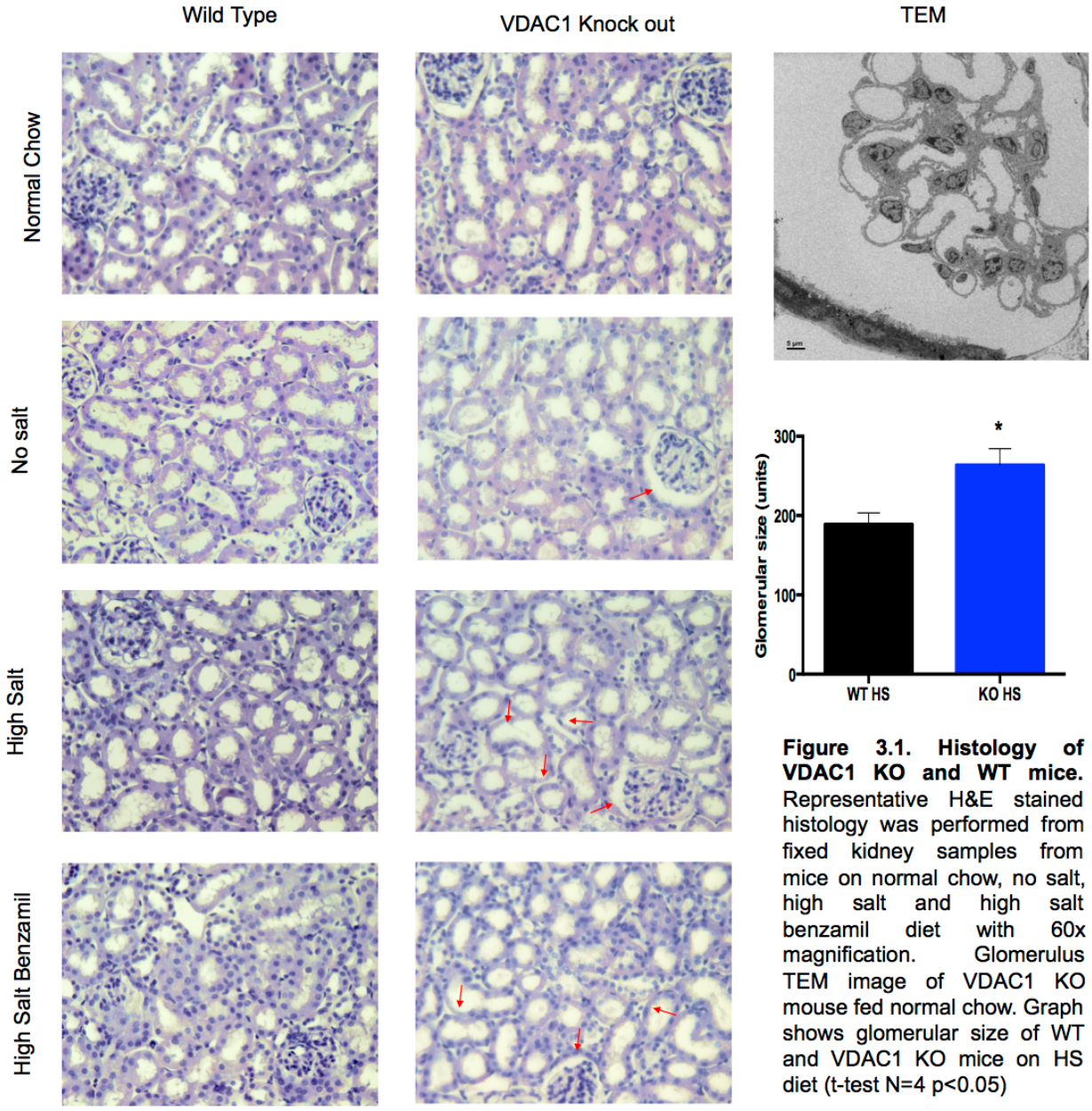


Figure 3.1. Histology of VDAC1 KO and WT mice. Representative H&E stained histology was performed from fixed kidney samples from mice on normal chow, no salt, high salt and high salt benzamil diet with 60x magnification. Glomerulus TEM image of VDAC1 KO mouse fed normal chow. Graph shows glomerular size of WT and VDAC1 KO mice on HS diet (t-test N=4 $p<0.05$)

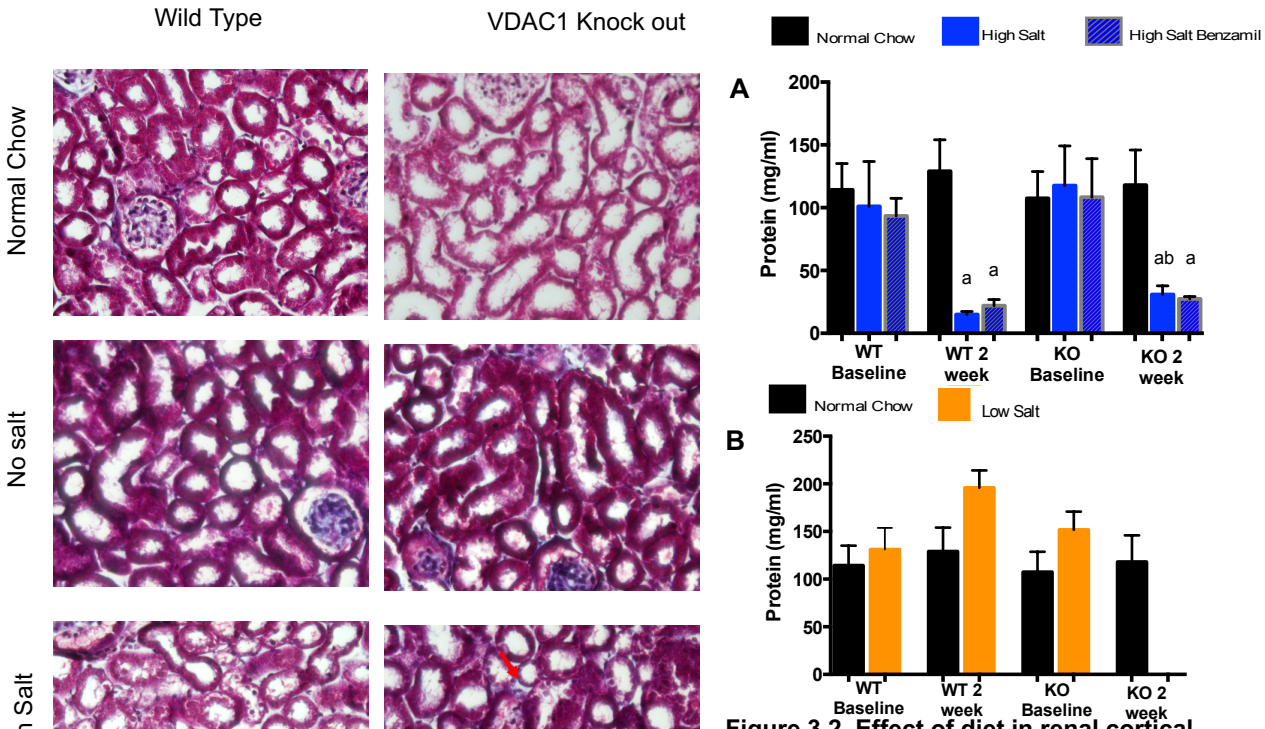


Figure 3.2. Effect of diet in renal cortical collecting duct. Representative images of sections after Masson's trichrome stain of renal cortex tissue under light microscope(60x) of wild type and VDAC1 knock out mice after two week treatment with different diets. Increases in collagen deposits around renal glomeruli (thick arrow) and tubules (thin arrows) 60x magnification **A.** Urine protein of wild type and VDAC1 KO mice was measured before and two weeks after high salt and high salt benzamil treatment. **B.** Urine protein of wild type and VDAC1 ko mice before and 2 weeks after no salt diet. Significant difference to normal chow is indicated by a, significant difference to same diet WT is indicated by b (ANOVA N=4 $p<0.05$)

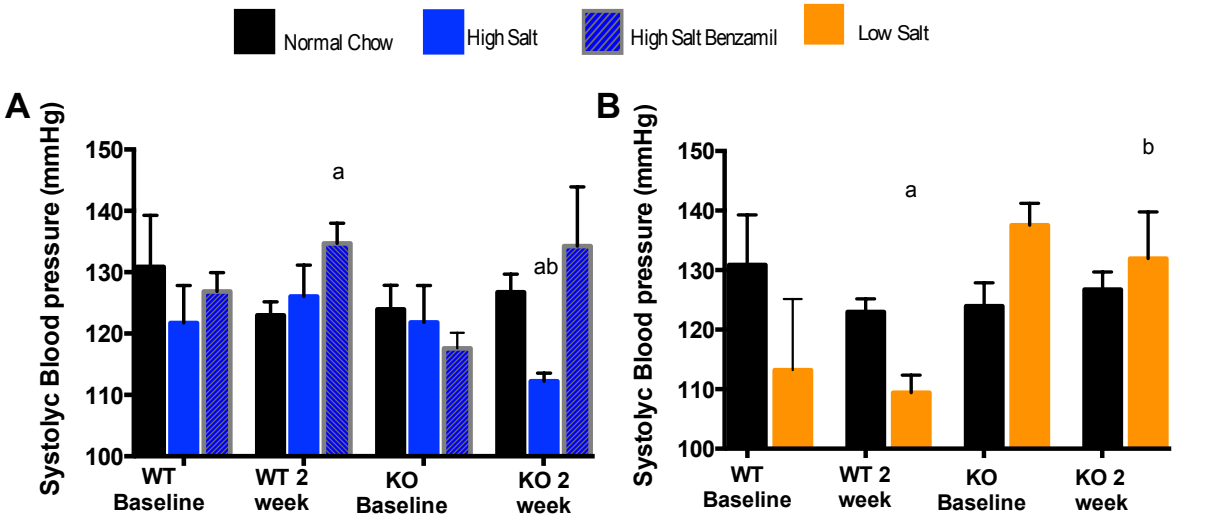


Figure 3.3. Systolic Blood pressure Wild type and VDAC1 Knock Out mice. WT and VDAC1 knock out mice were fed normal chow, no salt, high salt of high salt benzamil and urine osmolality was measured before and two weeks after treatment. Significant difference to normal chow is indicated by a, significant difference to same diet WT is indicated by b (ANOVA N=4 p<0.05)

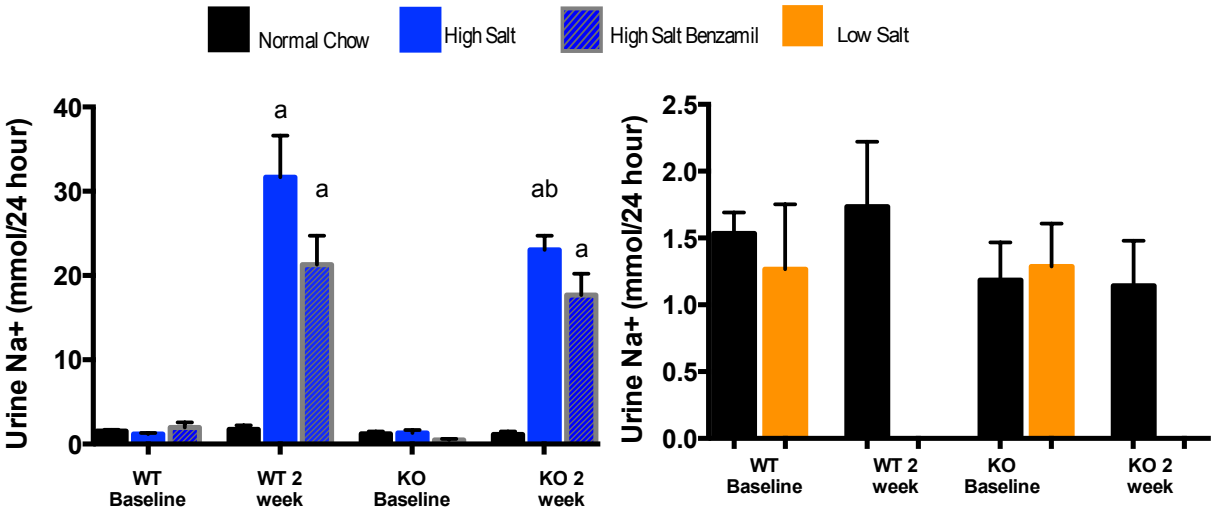


Figure 3.4. Urine Sodium in Wild type and VDAC1 Knock Out mice. WT and VDAC1 knock out mice were fed normal chow, no salt, high salt of high salt benzamil and urine sodium was measured before and two weeks after treatment. Significant difference to normal chow is indicated by a, significant difference to same diet WT is indicated by b (ANOVA N=4 p<0.05)

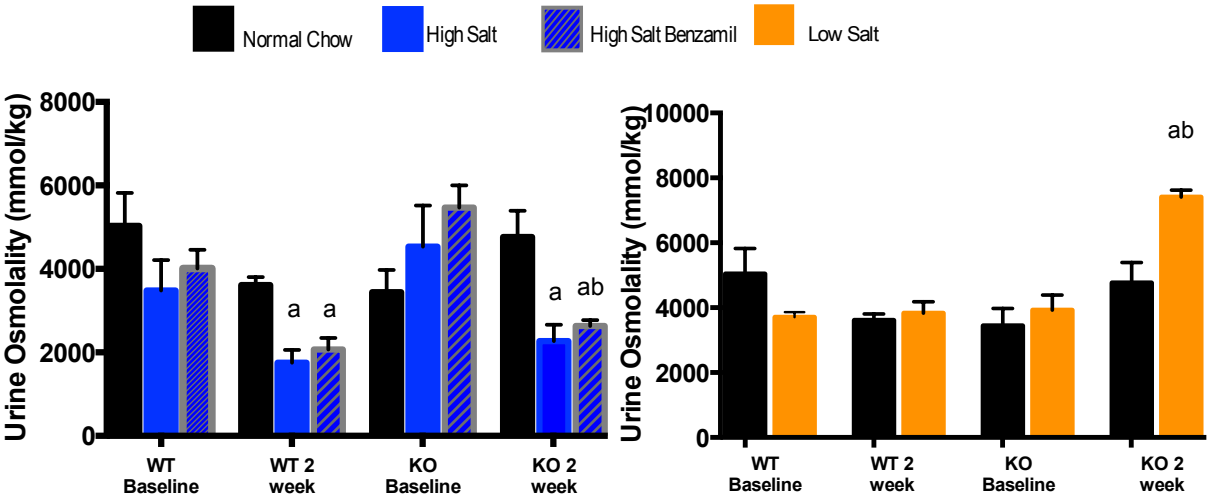


Figure 3.5. Urine Osmolality of Wild type and VDAC1 Knock Out mice. WT and VDAC1 knock out mice were fed normal chow, no salt, high salt of high salt benzamil and systolic blood pressure was measured before and two weeks after treatment. Significant difference to normal chow is indicated by a, significant difference to same diet WT is indicated by b (ANOVA N=4 $p<0.05$)

		Wild Type				VDAC 1 Knock Out			
Parameter	Week	Normal Chow	No Salt	High Salt	High Salt Benzamil	Normal Chow	Low salt	High Salt	High Salt Benzamil
Body Weight (g)	0	24.29±3.00	24.54±0.75	24.77±0.63	24.23±2.71	27.23±2.86	19.72±2.39	23.07±1.76	19.71±0.53
	2	22.72±2.22	22.85±0.66	22.54±0.312	21.61±1.41	24.25±2.79	18.32±1.87	21.33±1.70	16.64±0.77
Blood Pressure (mmHg)	0	130.9±8.41	130.9±8,407	121.7±6.09	126.9±3.06	124.0±2.91	137.5±3.65	121.8±5.99	117.6±2.524
	2	123.0±2.197	109.4±2.19	126.0±5.127	134.7±3.29[a]	126.7±2.96	131.9±7.825[b]	112.2±1.29[a,b]	134.2±9.66
Water intake (mL/ 24 hr)	0	2.86±1.23	3.44±1.16	3.13±0.59	4.77±0.41	3.46±0.62	3.82±0.87	3.52±0.40	2.95±0.32
	2	2.54±0.4	2.38±0.42	12.80±1.36[a]	8.76±0.86[a]	1.63±0.51	2.54±0.52	8.97±1.50[a,b]	5.05±0.51[a,b]
Food Intake (g/ 24 hr)	0	2.61±0.67	1.61±0.85	1.75±0.48	2.08±0.82	2.16±0.57	2.80±0.95	2.72±0.29	2.22±0.34
	2	2.41±0.35	2.36±0.26	3.78±0.56	2.38±0.28	1.19±0.44	2.49±0.6	2.98±0.18	2.17±0.43
Urine Output (mL/24 hr)	0	0.69±0.21	0.7±0.31	0.72±0.11	1.39±0.25	0.93±0.30	0.55±0.19	0.87±0.34	0.34±0.11
	2	0.94±0.37	0.28±0.11	8.90±0.98[a]	4.10±0.24[a]	0.61±0.12	0.05±0.05	3.99±0.70[a,b]	2.81±0.49[a]
Urine Sodium (mmol/24 hr)	0	1.735±0.484	1.917±0.166	1.184±0.129	1.973±0.586	1.186±0.281	2.431±0.460	1.320±0.348	0.488±0.126
	2	1.736±0.485	-	31.66±0.494[a]	21.29±0.343[a]	1.143±0.337	-	23.054±1.681[a,b]	17.670±2.532[a]
Urine Osmolality (mmol/kg)	0	5033±787	3703±159	3481±7331	4008±447	3443±545	3927±464	4535±9847	5470±530
	2	3615±379	3615±190[a]	1753±308[a]	2070±274[a]	4135±769	7412±212[a,b]	2273.3±390[a]	2633±142[a,b]
Urine Protein (mg/mL)	0	129±25.04	131±22.69	101.07±35.70	118±24.49	107.41±21.31	151.63±19.16	117.65±31.52	108.31±30.67
	2	114±20.89	147.42±50.09[a]	14.97±2.44[a]	22.04±4.81[a]	118.01±27.87	-	30.93±6.84[a,b]	27.4±1.80[a]

Table 3.1. Metabolic parameters of VDAC1 KO mice. WT and VDAC1 mice were fed normal chow, no salt, high salt of high salt benzamil and metabolic parameters were measured. Significant difference to normal chow is indicated by a, significant difference to same diet WT is indicated by b (ANOVA n=4 $p<0.05$)

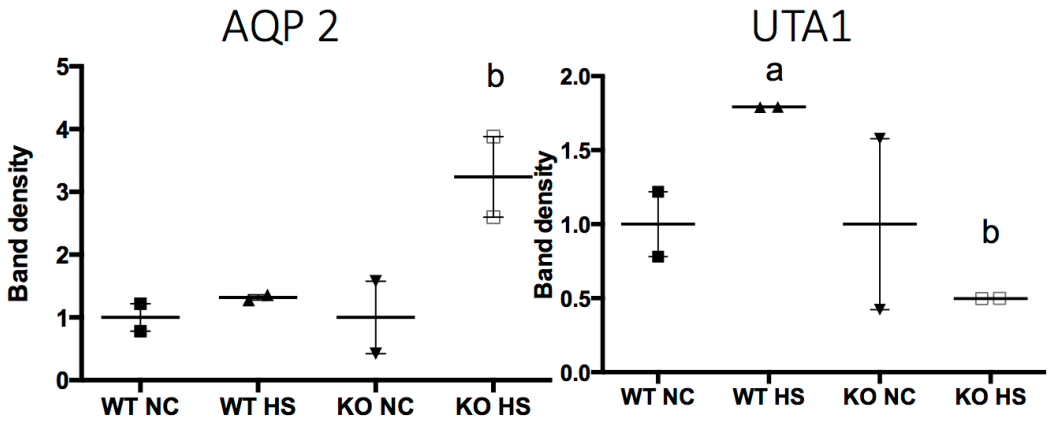


Figure 3.6. Aquaporin 2 and urea transporter (UT)-A1 protein abundance in VDAC1 KO mice on high salt diet. Density of bands was normalized to control. Significant difference to normal chow is indicated by a, significant difference to same diet WT is indicated by b (t-test N=2 $p<0.05$)

Parameter compared to wild type	
Blood pressure on high salt	↓
Blood pressure on low salt	↑
Urine output on high salt	↓↓
Water intake on high salt	↓
Urine Na^+ on HS	↑↑
Urine Osmolality on HS	↑
Urine Osmolality on low salt	↑↑
Urine Protein on HS	↑
Expression of AQP2	↑

Table 3.2. Summary of VDAC1 knock out metabolic parameters.

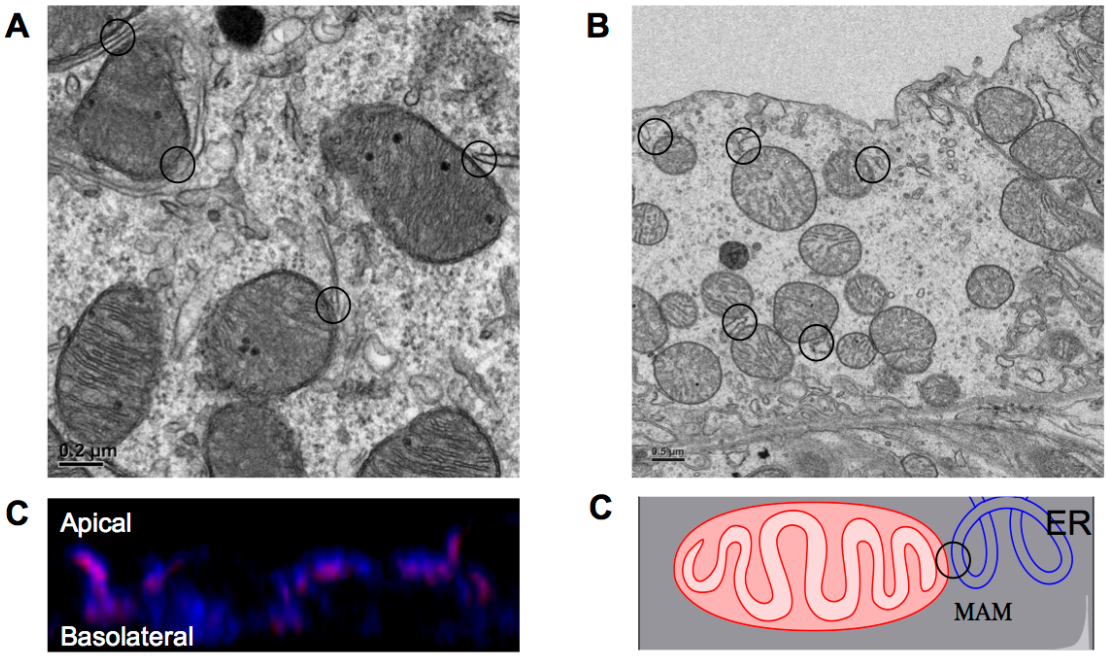


Figure 4.1. Mitochondria ER association. A. wild type mouse principal cell B. VDAC1 knock out mouse principal cell C. mpkCCD cell stained with mitotrackerRed (red) and ERtracker (blue) D. schematic of close contact between mitochondria and ER

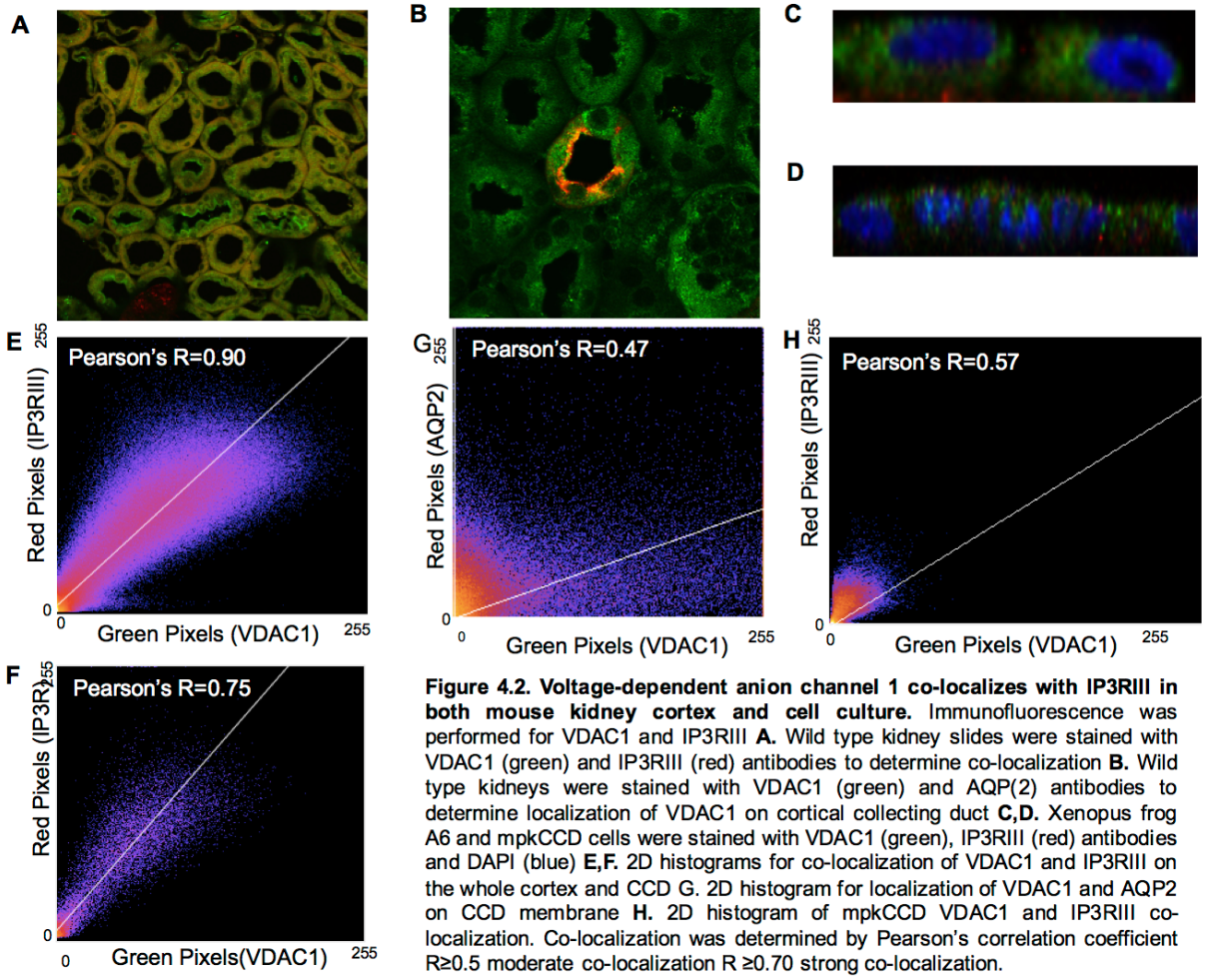


Figure 4.2. Voltage-dependent anion channel 1 co-localizes with IP3RIII in both mouse kidney cortex and cell culture. Immunofluorescence was performed for VDAC1 and IP3RIII **A.** Wild type kidney slides were stained with VDAC1 (green) and IP3RIII (red) antibodies to determine co-localization **B.** Wild type kidneys were stained with VDAC1 (green) and AQP(2) antibodies to determine localization of VDAC1 on cortical collecting duct **C,D.** Xenopus frog A6 and mpkCCD cells were stained with VDAC1 (green), IP3RIII (red) antibodies and DAPI (blue) **E,F.** 2D histograms for co-localization of VDAC1 and IP3RIII on the whole cortex and CCD G. 2D histogram for localization of VDAC1 and AQP2 on CCD membrane **H.** 2D histogram of mpkCCD VDAC1 and IP3RIII co-localization. Co-localization was determined by Pearson's correlation coefficient R≥0.5 moderate co-localization R ≥0.70 strong co-localization.

References:

1. Soundararajan, R., M. Lu, and D. Pearce, *Organization of the ENaC-regulatory machinery.* Crit Rev Biochem Mol Biol, 2012. **47**(4): p. 349-59.
2. Gehlert S, Bloch W, Suhr F. Ca^{2+}-Dependent Regulations and Signaling in Skeletal Muscle: From Electro-Mechanical Coupling to Adaptation. International journal of molecular sciences. 2015;16(1):1066-95. doi: 10.3390/ijms16011066. PubMed PMID: 25569087.
3. Schneggenburger R, Han Y, Kochubey O. Ca($^{2+}$) channels and transmitter release at the active zone. Cell Calcium. 2012;52(3-4):199-207. doi: 10.1016/j.ceca.2012.04.011. PubMed PMID: 22682961.
4. Vasington, F. D. & Murphy, J. V. Ca ion uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation. *J. Biol. Chem.* **237**, 2670–2677 (1962).
5. Deluca, H. F. & Engstrom, G. W. Calcium uptake by rat kidney mitochondria. *Proc. Natl Acad. Sci. USA* **47**, 1744–1750 (1961)
6. Lehninger, A. L., Rossi, C. S. & Greenawalt, J. W. Respiration-dependent accumulation of inorganic phosphate and Ca ions by rat liver mitochondria. *Biochem. Biophys. Res. Commun.* **10**, 444–448 (1963).
7. Vance JE. Phospholipid sunthesis in a membrane fraction associated with mitochondria. K. Biol Chem. **265**, 7248-56 (1990)
8. Rizzuto, R. *et al*. Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. *Science* **280**, 1763–1766 (1998). PubMed PMID: 9624056

9. Giorgi, C. *et al.* Mitochondria-Associated Membranes: Composition, Molecular Mechanisms and Physoppathological Implications. *Antioxid, Redox Signal.* **22,** 995-1019 (2015). PubMed: PMID: 25557408

10. Hajnóczky G , Csodrdás G and Yi M. *Old players in a new role: mitochondria-associated membranes, VDAC, ryanodine receptors as contributors to the calcium signal propagation from endoplasmic reticulum to the mitochondria*. Cell Calcium, 2002. **32**(5-6): p. 363-77.

11. Szabadkai G. et al. *Chaperone-mediated coupling of endoplasmic reticulum and mitochondria Ca2+ channels.* K Cell Biol, 2006. **175**(6): p. 901-11.

12. Tubbs, E., et al., *Mitochondria-associated endoplasmic reticulum membrane (MAM) integrity is required for insulin signaling and is implicated in hepatic insulin resistance.* Diabetes, 2014. **63**(10): p. 3279-94.

13. Rizzuto R, De Stefani D, Raffaello A, Mammucari. Mitochondria as sensors and regulators of calcium signalling. 2012 Nat Rev Mol Cell Biol 13:566–578

14. Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, Tuft RA, Pozzan T (1998) Close contacts with the endo- plasmic reticulum as determinants of mitochondrial Ca2+ responses. Science 280:1763–1766

15. Csordas G, Thomas AP, Hajnoczky G Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria. 1999 EMBO J 18:96–108

16. Wang L, Yang X and Shen Y. Molecular mechanism of mitochondrial calcium uptake. Springer Cell Mol. Life Sci, 2015. 72:1489-1498

17. Dunn K, Kamocja M, McDonald J. A practical guide to evaluationg colocalization in biological microscopy. AM J. Physiol Cell Physiol. 2011. 300(4) C723-742. PMCID 3074624

18. Thai TL, Yu L, Galarza-Paez L, Wu M, Lam HY, Bao HF, Duke BJ, Al-Khalili O, Ma H, Liu C, Eaton DC. *Polarized effect of intracellular calcium on the renal epithelial sodium channel occurs as a result of subcellular calcium signaling domains maintained by mitochondria.* JBC, 2015. 290 (48):28805-11. PMCID4661396

19. Li Z, Liu Y, Yang L, Wang S, Guan MX. Maternally inherited hypertension is associated with the mitochondrial tRNA(Ile) A4295G mutation in a Chinese family. Biochem Biophys Res Commun. 2008;367(4):906-11. doi: 10.1016/j.bbrc.2007.12.150. PubMed PMID: 18177739.

20. Blessing NW, Blount MA, Sands JM, Martin CF, Klein JD. Urea transporters UT-A1 and UT-A3 accumulate in the plasma membrane in response to increased hypertonicity. Am J Physiol Renal Physiol 295: F1336–F1341, 2008

21. Chou CL, Sands JM, Nonoguchi H, Knepper MA. Concentration dependence of urea and thiourea transport in rat inner medullary collecting duct. Am J Physiol Renal Fluid Electrolyte Physiol 258: F486–F494, 1990.

22. Sands JM, Schrader DC. An independent effect of osmolality on urea transport in rat terminal inner medullary collecting ducts. J Clin Invest 88: 137–142, 1991.

23. Lawen Alfons. Apoptosis- an introduction. Bio Essays, 2003. 25: 888-896. DOI: 10.1002/bies.10329

24. Brahimi-Horn CM et al. Knock out of Vdac1 activates hypoxia-inducible factor reactive oxygen species generation and induces tumor growth by promoting metabolic reprogramming and inflammation. Cancer and metabolism, 2015. 3:8 DOI: 10.1186/s40170-015-0133-5

25. Rossier, B. C. (2014) Epithelial sodium channel (ENaC) and the control of blood pressure. *Current Opinion in Pharmacology* **15**, 33-46

26. Su, Y. R., and Menon, A. G. (2001) Epithelial sodium channels and hypertension. *Drug metabolism and disposition: the biological fate of chemicals* **29**, 553-556

27. Laffer, C. L., Elijovich, F., Eckert, G. J., Tu, W., Pratt, J. H., and Brown, N. J. (2014) Genetic variation in CYP4A11 and blood pressure response to mineralocorticoid receptor antagonism or ENaC inhibition: an exploratory pilot study in African Americans. *Journal of the American Society of Hypertension : JASH* **8**, 475-480

28. Kashlan, O. B., and Kleyman, T. R. (2012) Epithelial Na(+) channel regulation by cytoplasmic and extracellular factors. *Experimental cell research* **318**, 1011-1019

29. Busst, C. J. (2013) Blood pressure regulation via the epithelial sodium channel: from gene to kidney and beyond. *Clinical and experimental pharmacology & physiology* **40**, 495-503

30. Schaeffer, C., Creatore, A., and Rampoldi, L. (2014) Protein trafficking defects in inherited kidney diseases. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* **29 Suppl 4**, iv33-44

31. Toney, G. M., Vallon, V., and Stockand, J. D. (2012) Intrinsic control of sodium excretion in the distal nephron by inhibitory purinergic regulation of the epithelial Na(+) channel. *Current opinion in nephrology and hypertension* **21**, 52-60

32. Vallon, V., and Rieg, T. (2011) Regulation of renal NaCl and water transport by the ATP/UTP/P2Y2 receptor system. *Am J Physiol Renal Physiol* **301**, F463-475

33. Bao, H. F., Thai, T. L., Yue, Q., Ma, H. P., Eaton, A. F., Cai, H., Klein, J. D., Sands, J. M., and Eaton, D. C. (2014) ENaC activity is increased in isolated, split-open cortical collecting ducts from protein kinase Calpha knockout mice. *Am J Physiol Renal Physiol* **306**, F309-320

34. Pochynyuk, O., Bugaj, V., Vandewalle, A., and Stockand, J. D. (2008) Purinergic control of apical plasma membrane PI(4,5)P2 levels sets ENaC activity in principal cells. *American journal of physiology. Renal physiology* **294**, F38-46

35. Yue, G., Malik, B., Yue, G., and Eaton, D. C. (2002) Phosphatidylinositol 4,5-bisphosphate (PIP2) stimulates epithelial sodium channel activity in A6 cells. *The Journal of biological chemistry* **277**, 11965-11969

36. Alli, A. A., Bao, H. F., Alli, A. A., Aldrugh, Y., Song, J. Z., Ma, H. P., Yu, L., Al-Khalili, O., and Eaton, D. C. (2012) Phosphatidylinositol phosphate-dependent regulation of Xenopus ENaC by MARCKS protein. *American journal of physiology. Renal physiology* **303**, F800-811

37. Henry, P. C., Kanelis, V., O'Brien, M. C., Kim, B., Gautschi, I., Forman-Kay, J., Schild, L., and Rotin, D. (2003) Affinity and specificity of interactions between Nedd4 isoforms and the epithelial Na+ channel. *The Journal of biological chemistry* **278**, 20019-20028

38. Thai, T. L., Yu, L., Eaton, D. C., Duke, B. J., Al-Khalili, O., Lam, H. Y., Ma, H., and Bao, H. F. (2014) Basolateral P2X(4)channels stimulate ENaC activity in Xenopus cortical collecting duct A6 cells. *Am J Physiol Renal Physiol* **307**, F806-813

39. Zhang, Y., Sanchez, D., Gorelik, J., Klenerman, D., Lab, M., Edwards, C., and Korchev, Y. (2007) Basolateral P2X4-like receptors regulate the extracellular ATP-stimulated epithelial Na+ channel activity in renal epithelia. *American journal of physiology. Renal physiology* **292**, F1734-1740

40. Ribeiro, C. M., Paradiso, A. M., Livraghi, A., and Boucher, R. C. (2003) The mitochondrial barriers segregate agonist-induced calcium-dependent functions in human airway epithelia. *J Gen Physiol* **122**, 377-387

41. Tinel, H., Cancela, J. M., Mogami, H., Gerasimenko, J. V., Gerasimenko, O. V., Tepikin, A. V., and Petersen, O. H. (1999) Active mitochondria surrounding the pancreatic acinar granule region prevent spreading of inositol trisphosphate-evoked local cytosolic Ca(2+) signals. *EMBO J* **18**, 4999-5008

42. Bao, H. F., Song, J. Z., Duke, B. J., Ma, H. P., Denson, D. D., and Eaton, D. C. (2012) Ethanol stimulates epithelial sodium channels by elevating reactive oxygen species. *American journal of physiology. Cell physiology* **303**, C1129-1138

43. Shigetomi, E., Kracun, S., Sofroniew, M. V., and Khakh, B. S. (2010) A genetically targeted optical sensor to monitor calcium signals in astrocyte processes. *Nature neuroscience* **13**, 759-766

44. Trollinger, D. R., Cascio, W. E., and Lemasters, J. J. (1997) Selective loading of Rhod 2 into mitochondria shows mitochondrial Ca2+ transients during the contractile cycle in adult rabbit cardiac myocytes. *Biochem Biophys Res Commun* **236**, 738-7443

45. Finkel, T., Menazza, S., Holmstrom, K. M., Parks, R. J., Liu, J., Sun, J., Liu, J., Pan, X., and Murphy, E. (2015) The ins and outs of mitochondrial calcium. *Circ Res* **116**, 1810-1819

46. Gincel, D., Vardi, N., and Shoshan-Barmatz, V. (2002) Retinal voltage-dependent anion channel: characterization and cellular localization. *Investigative ophthalmology & visual science* **43**, 2097-2104

47. Kirichok, Y., Krapivinsky, G., and Clapham, D. E. (2004) The mitochondrial calcium uniporter is a highly selective ion channel. *Nature* **427**, 360-364

48. Wildman, S. S., Kang, E. S., and King, B. F. (2009) ENaC, renal sodium excretion and extracellular ATP. *Purinergic Signal* **5**, 481-489

49. Farah, C. A., and Sossin, W. S. (2012) The role of C2 domains in PKC signaling. *Advances in experimental medicine and biology* **740**, 663-683

50. Bao, H. F., Zhang, Z. R., Liang, Y. Y., Ma, J. J., Eaton, D. C., and Ma, H. P. (2007) Ceramide mediates inhibition of the renal epithelial sodium channel by tumor necrosis factor-alpha through protein kinase C. *American journal of physiology. Renal physiology* **293**, F1178-1186

51. Wang, J., Peng, Q., Lin, Q., Childress, C., Carey, D., and Yang, W. (2010) Calcium activates Nedd4 E3 ubiquitin ligases by releasing the C2 domain-mediated auto-inhibition. *The Journal of biological chemistry* **285**, 12279-12288

52. Gilmore, E. S., Stutts, M. J., and Milgram, S. L. (2001) SRC family kinases mediate epithelial Na+ channel inhibition by endothelin. *The Journal of biological chemistry* **276**, 42610-42617

53. Snyder, P. M. (2002) The epithelial Na+ channel: cell surface insertion and retrieval in Na+ homeostasis and hypertension. *Endocrine reviews* **23**, 258-275

54. Hill, W. G., Butterworth, M. B., Wang, H., Edinger, R. S., Lebowitz, J., Peters, K. W., Frizzell, R. A., and Johnson, J. P. (2007) The epithelial sodium channel (ENaC) traffics to apical membrane in lipid rafts in mouse cortical collecting duct cells. *The Journal of biological chemistry* **282**, 37402-37411

55. Williams, G. S., Boyman, L., Chikando, A. C., Khairallah, R. J., and Lederer, W. J. (2013) Mitochondrial calcium uptake. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 10479-10486

56. Brustovetsky, N., Brustovetsky, T., Jemmerson, R., and Dubinsky, J. M. (2002) Calcium-induced cytochrome c release from CNS mitochondria is associated with the permeability transition and rupture of the outer membrane. *Journal of neurochemistry* **80**, 207-218

57. Liu, Y., Gao, L., Xue, Q., Li, Z., Wang, L., Chen, R., Liu, M., Wen, Y., Guan, M., Li, Y., and Wang, S. (2011) Voltage-dependent anion channel involved in the mitochondrial calcium cycle of cell lines carrying the mitochondrial DNA A4263G mutation. *Biochem Biophys Res Commun* **404**, 364-369.

58. Tang, Y. & Zucker, R. S. Mitochondrial involvement in post-tetanic potentiation of synaptic transmission. *Neuron* **18**, 483–491 (1997).

59. Billups, B. & Forsythe, I. D. Presynaptic mitochondrial calcium sequestration influences transmission at mammalian central synapses. *J. Neurosci.* **22**, 5840–5847 (2002)

60. Swift PA and Macgregor GA. Genetic variation in epithelial sodium channel: a risk facor for hypertension in people of African origin. Adv. In Renal Replacement Therapy. 11(1). 76-86 (2004).

61. De Stefani D, Bononi A, Romagnoli A, Messina A, De Pinto V, Pinton P, Rizzuto R. Vdac1 selectivity transfers apoptotic Ca^{2+} signals to mitochondria. *Cell Death Differ*. 19:267-273 (2012)

62. Ma HP, Saxena S, Warnock DG. Anionic phospholipids regulate native and expressed epithelial sodium channel (ENaC). *J Biol Chem*. 2002 Mar 8; 277(10):7641-4.

63. Pochynyuk O, Bugaj V and Stockland JD. Physiologic regulation of the epithelial Na^{+} channel (ENaC) by phosphotidylinositides. *Curr Opin Neprhol Hypertens*. 17(5):533-540 (2008).

64. Bootman MD and Berridge MJ The elemental principles of calcium signalling. Cell 83: 675–678(1995).

65. Clapham DE Calcium signalling. Cell 80: 259–268(1995).

66. Bootman MD, Lipp P. Calcium Signaling and regulation of Cell Function. Encyclopedia of life sciences.(2001)

67. Ford P, Rivarola V, Chora O, Blot-Chabaud M, Cluzeaud F, Farman N, Parisi M and Capurro C. Volume regulation in cortical collecting duct cells: role of AQP2. *Biol Cell* 97(9) 687-97 (2005).

68. Tang Y and Stephenson JL. Calcium dynamics and homeostasis in a mathematical model of the principal cell of the cortical collecting tubule. *J Gen Physiol.* 107(2) 207-30 (1996).

69. Mistry AC, Mallick R, Fröhlich O, Klein JD, Rehm A, Chen G and JM sands. The UT-A1 Urea Transporter Interacts with Snapin, a SNARE-associated Protein. *JBC* 282, 30097-30106 (2007). doi: 10.1074/jbc.M705866200

70. Wai Timothy and Langer Thomas. Mitochondrial Dynamics and Metabolic regulation. Trends in Endocrinology & Metabolism. 27(2) 105-117 (2016)

71. Vito De Pinto, Angela Messina, Darius J.R. Lane, Alfons Lawen. Voltage-dependent anion-selective channel (VDAC) in the plasma membrane. *FEBS letters* 584(9) 1793-1799(2010).

72. Per Svenningsen, James L. Buford and János Peti-Peterdi. ATP Releasing Connexin 30 Hemichannels Mediate Flow-Induced Calcium Signaling in the Collecting Duct. *Front Physiol.* 4:292. doi: 10.3389/fphys.2013.00292 (2013).