PROTEIN PARTICIPANTS OF CYTOSOLIC INTERNALIZATION OF THE OUABAIN-BOUND NA⁺/K⁺ATPASE RECEPTOR IN HUMAN B-3 LENS EPITHELIAL CELLS

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Joshua Lysle Stricker ENTITLED Protein Participants of Cytosolic Internalization of the Ouabain-bound Na⁺/K⁺ATPase Receptor in Human B-3 Lens Epithelial Cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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The primary function of Na+/K+ATPase (NKA) discovered by Jens Christian Skou in 1958, through electrogenic exchange of 3 Na⁺ for 2 K⁺ ions (Robert L Post, 1958), is the homeostatic preservation of their electrochemical gradient across the plasma membrane (PM) of almost all species and their derived cell lines. Cardiotonic steroids (CTS), such as cardenolides or bufadienolides, are well established inhibitors of NKA. Fox glove plant extracts containing CTS, including digitalis, were first described by William Withering (1744-91) for their positive inotropic effect in heart failure patients. Later research identified the mechanism of their action through direct inhibition of NKA with simultaneous cytosolic Na⁺ accumulation and due to reversal of the Na⁺/Ca²⁺ exchanger located in series with NKA in the plasma membrane. Subsequent increases in cytoplasmic Ca²⁺ levels lead to higher contractility of the heart, the inotropic effect of CTS. The Strophantus gratus-derived CTS ouabain is experimentally widely used. During the past two decades, ouabain binding to NKA, resulting in the here so coined \textit{NKA-ouabain receptor complex} (NORC), has been shown to elicit at least two signaling pathways involving protein kinases with sequential phosphorylation/transphosphorylation reactions leading to transcriptional upregulation of proteins
involved in cell proliferation, survival, and death. More recent studies suggest NORC internalization through the plasmalemmal membrane and its cytosolic appearance. Mechanisms of NORC internalization and ensuing putative actions of NKA or ouabain or both have yet to be identified. In the present work, it was hypothesized that during its membrane to cytosol descent, NORC utilizes a variety of cytosolic and cytoskeletal proteins through protein/protein interactions as an escalator to reach endosomes and lysosomes for subsequent breakup into its NKA receptor and ligand ouabain. NORC internalization would then serve as an alternate model to explain novel transcriptional gene upregulation pathways which are different from early protein/protein interaction-based kinase upregulation. In the present work, human lens epithelial (B-3) cells were used as an experimental model, where the presence of only NKA α1 isoform was established by RTPCR, and the membrane transport properties have been well defined (Lauf et al., 2006). Proteins potentially interacting with NKA, such as the oncogenic protein BclXL (B-cell lymphoma derived), α-tubulin, caveolin-1, EEA1 (endosome-associated antigen), and LAMP1 (lysosomal-associated membrane protein) in the presence and absence of ouabain, were analyzed by SDS-electrophoresis and Western Blot. Colocalization and cytosolic internalization of NORC by Bodipy-fluorescent ouabain (BFO) and anti-α1 specific Cy3-red labeled antibodies (AB) is presented by immunocytochemical evidence. B-3 cells incubated with unlabeled ouabain at > nanomolar concentrations showed increases in cell size and redistribution of Cy3-labeled anti-α1 AB, signifying ouabain-induced cell swelling presumably due to Na⁺ and water entry. Anti-α1 AB Cy3-red labeled NORC colocalization with Alexa-Fluor green marked AB against, α-tubulin increased at higher ouabain concentrations, while decreasing with
acetylation resulting from exposure to the histone deacetylase inhibitor Tricostatin
(TSA). As expected, NKA also colocalized with caveolin-1 and with EEA1 and LAMP1.
These findings suggest that NORC-colocalizing proteins provide a rough outline of its
cytosolic fate and form the basis for a preliminary model delineating its cytosolic fate.
This information may contribute to our understanding of clinically observed attenuating
effects of CTS in cancer biology and chronic kidney disease (CKD).
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List of Abbreviations

NKA – Sodium Potassium ATPase
ATP – Adenosine Triphosphate
CTS – Cardiotonic Steroids
NORC – Na⁺/K⁺ATPase ouabain receptor complex
Na⁺ - Sodium ion
K⁺ - Potassium ion
Ca²⁺ – Calcium ion
BFO – BODIPY Fluorescent Ouabain
CO – Unlabeled ouabain
Bcl-2 - B-cell lymphoma – 2
Bcl-XL – B-cell lymphoma – extra large
Bcl-XS – B-cell lymphoma – extra small
LAMP1 – Lysosomal associated membrane protein 1
EEA1 – Early endosomal antigen 1
MT – Microtubules
Oac-Tubulin – Acetylated tubulin
SRC-1 – Steroid Receptor Coactivator-1
SRC-3 – Steroid Receptor Coactivator-3
RT-PCR – Reverse transcriptase-polymerase chain reaction
**WB** – Western blot

**ICC** – Immunocytochemistry

**AB** - Antibody

**R** – Receptor

**TSA** – Tricostatin-A
I. INTRODUCTION AND BACKGROUND

The Na\textsuperscript{+}/K\textsuperscript{+} ATPase (NKA)

The ubiquitously expressed Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (NKA) belongs to the P-type ATPases. According to the chemi-osmotic theory (Mitchell 1961), its principal role is to maintain the ionic gradients of sodium (Na\textsuperscript{+}) and potassium (K\textsuperscript{+}) across the plasmalemma through the utilization of energy derived from hydrolysis of adenosine triphosphate (ATP) for active transport of the ions. Through active redistribution of ions, cells maintain, on average, a -70 mV resting membrane potential. Resting potentials for cells were first described by Walther Nernst whose contributions brought about the Nernst equation. K\textsuperscript{+} ions due to their lower equilibrium potential (Nernst Potential $E = -91 \text{ mV}$) naturally flow out of cells as they are more negative than the transmembrane potential (Suhail, 2010), whereas Na\textsuperscript{+} ions are driven into the cell from a large extracellular pool. NKA’s P-type classification refers to phosphorylation-dependent conformational changes it undergoes between E\textsubscript{1} and E\textsubscript{2} during its canonical cycle (the Post-Albers Cycle), E\textsubscript{1} for the active movement of 3Na\textsuperscript{+} ions out of the cell, and E\textsubscript{2} for 2K\textsuperscript{+} ions into the cytosol (Post et al., 1960). The electrochemical potential gradients created for both ion species are necessary for cellular functions such as secondary active transport, excitability, and volume regulation (Apell 2014). Without these functions eukaryotic life would not be sustainable. As Sodium ions are replace potassium, they are considered toxic. This replacement prevents key interactions necessary during biosynthesis preventing proper function of some specific proteins (Garcia-Salcedo, 2006). Their increase has been
related to transcriptional upregulation (Xie et al., 2001). Therefore, NKA due to its ubiquitous and essential nature, has become a prevalent target for emerging pharmaceuticals (Yatime et al., 2009). Their disruption of the electrochemical gradient, as some studies have shown, has also been related to improved cancer treatment outcomes when treated in tandem with ouabain Cardiac glycosides (Garcia, 2015). Hypertension, salt disequilibria, programmed cell death, cardiovascular, neurological, and renal disorders, maturation of spermatozoons, volume regulation, rheumatoid arthritis, sepsis, familial hemiplegic migraine type 2, lung edema clearance, rapid-onset dystonia-Parkinsonism and preeclampsia have all been associated one way or another to NKA function or dysfunction (Suhail, 2010, Geering, 2006). As NKA activity influences such a broad range of ailments, this suggest regulatory mechanisms associated with the interaction with cardiac glycosides.

NKA is comprised of α and β subunits forming a heterodimer. The α isoform, is the catalytic unit required for ion transport, existing in four isoforms (α1–α4) whereas three β subunit isoforms (β1–β3) have been identified thus far. One of seven γ subunits from the family of the FXYD (phenylalanine-X-tyrosine-aspartate) proteins (Geering, 2006) is involved in Na pump regulation by through in interaction of α and β subunits thus forming functionally a trimeric oligomer (Dietze, 2013). NKA α subunits are multi-spanning transmembrane proteins responsible for the catalytic activity of the ATPase and transport properties. α subunits also provide a well-documented binding site for cations, ATP, and cardiotonic steroids (CTS) (Pierre, 2006). CTS’s have been in use for over two centuries from a clinical and research perspective. Due to their pharmacologically known selectivity, CTS can be employed to identify how a wide variety of cells react when they target NKA and form the NKAR-CTS complex (NORC).
**Cardiotonic Steroids (Ouabain)**

CTS compounds have a generic structure which consists of a steroid core and a lactone ring which constitutes pharmacophoric moiety responsible for their activity. Cardiac Glycosides generic structure contains a steroid core which is double-substituted with an unsaturated 5 or 6 member lactone ring and carbon 17. A sugar moiety is associated with the position 3 carbon (Prassas et al., 2008). Structural differentiations are shown in Figure 1.1 identifying of cardenolides vs. bufadienolides based on the 5 or 6-member lactone ring.

![Generic structural composition of cardiotonic steroids](image)

**Figure 1.1. Generic structural composition of cardiotonic steroids**

The figure above breaks down the two known subsets of cardiotonic steroids and their core components. The “A, B, C, and D” internal cycloperhydrophenanthrene rings comprising the steroid core, “R” the sugar group, and “L” the buta-lactone or pyrone rings which are determinants of the cardenolide or bufadienolide structure of CTS, respectively (Prassas, et al., 2008).
Digitalis purpurea extracts were first utilized by, William Withering in 1785, when treating cardiac disorders, as they contained cardiac glycosides (Foszzard, 1985). This type of pharmacological regimen was known for thousands of years through the emerging cultures in the old and new worlds. (Schatzmann, 1953). Schatzmann identified the mechanism of cardiac glycosides action as specific NKA inhibitors in red blood cells. Clinical studies have shown positive results of an orally administered ouabain when treating a variety of cardiovascular diseases (Fürstenwerth et al., 2010). Ouabain has been used for decades in both research and clinical treatments. When ouabain binds to NKA, NORC is formed and ion exchange is inhibited leading to a buildup of cellular Na⁺ concentrations, \([\text{Na}^+]_i\), causing reversal of the Na⁺/Ca²⁺ exchanger through Na⁺ ion outflow and Ca²⁺ ion inflow generating an overall rise of intracellular Ca²⁺ (Shattock et al, 2015). In cardiac myocytes, increased Ca²⁺ ions produce a positive inotropic effect, i.e. an increase in heart muscle contractions, providing the necessary conditions required for increased stroke volumes of the heart for patients undergoing congestive heart failure (Schoner et al., 2007).

In additional studies, Ouabain’s role was established in apoptosis based on cell specificity. For example, ouabain has shown pro-apoptotic activity in the following cell types: “normal neuronal, neuro- and glioblastoma, spiral ganglion neurons, hepatic, blood peripheral lymphocytes, lymphoma, prostate cancer and cochlear hair cells” (Venugopal & Blanco, 2016, Yong, 2013). Apoptosis is often governed by B-cell lymphoma 2 (BCL-2) proteins which through caspases and cysteine protease activity molecular cascades cause cell death (Venugopal & Blanco, 2016). Regulatory activity of Bcl-2 proteins and Ca²⁺ by
NKA could be indicative of a protective mechanism through interactions with endogenous ouabain in a previously unidentified pathway involving reduced activity of the receptor.

**Bcl-2 Protein families**

The Bcl-2 protein group, such as pro-survival Bcl-2, Bcl-XL, and Mcl-1 proteins and pro-apoptotic proteins including Bak, Bax, BIM and Bid. This family of proteins control a pathway in which programmed cell death occurs (Strasser et al., 2002, Schendel et al., 2000). Differing isoforms within protein family are broken down and organized into their pro- or anti-apoptotic subsets per organization of differing motifs within each group. “Bcl-2 protein structures have four homology domains (BH 1-4). These domains (BH1-4) dictate protein activity, which through mutual BH1/BH3 domain interactions determine the pro-survival or pro-apoptotic outcome of a cell (Wolf, 2006). To the conflicting pro-and anti-apoptotic Bcl-2 protein groups maintain. For example, BAK and BAX, prevent homodimerization to pro-apoptotic mitochondrial pore forming structures which result in cell death (Lauf et al., 2014) (shown in Figure 1.2). These natural cell death regulatory mechanisms have attracted great interest as plausible specific treatment opportunities for several cancers. Through modification of expression, and cytosolic availability of these proteins, the survivability of the cell can be directly altered.
Figure 1.2. Model depicting Bcl-2 protein to protein interactions leading to pro/anti-apoptotic conditions within cell.

Model showing the differing interactions between pro-apoptotic and anti-apoptotic proteins and their possible conjugated configurations resulting in pro-survival BH-1 and BH-3 interaction and inhibition, or activation through homodimerization of BH-3 motif proteins which causes cell death.

One proposal combines the relationship between Bcl-2 protein cytosolic concentrations and Bcl-2 interactions possibly altered through their reciprocal interaction with NKA. This interaction would occur due to BH-1 and BH-3 motifs, previously identified upon sequence analysis of crystalized NKA (Lauf et al., 2013). These putative motifs found on NKA could interact directly with pro- and anti-apoptotic Bcl-2 subfamilies thereby having a direct effect on cell signaling and survival. In conjunction with this theory, a research group in Sweden showed that in rat proximal tubule cells, ouabain attenuated the imbalance between the pro-apoptotic effects of BAX and its inhibitor Bcl-XL (Burlaka et al., 2013). We propose CTS-driven effects correspond with NKA inhibition and NKA’s Bcl-2 motifs and possible unveiling of BH-3 motifs that interact within the cell.

Caveolin-1

Caveolae are flask-shaped, noncoated vesicular membrane invaginations containing 21 to 24 kDa membrane proteins act as scaffolding to maintain the structural associated components of caveolae (Liu et al., 2004). Caveolae are known constituents of endocytotic actions with three known groups of genes expressed in mammals (caveolin-1, -2, and -3), and currently five known protein isoforms. NKA endocytosis in proximal tubule cells was found to only be initiated in the presence of caveolin-1 (Liu et al., 2002,
Caveolin typically functions as scaffolding for lipid transport interacting with cholesterol creating lipid rafts, which are transported to the endoplasmic reticulum. Caveolin-1 attracts proteins to caveolae similarly to clathrin adaptors in the way they attract transmembrane receptors to coated pits and function as a molecular motors for membrane invagination and budding (Liu et al., 2002). Ouabain induces endocytosis of membrane bound NKA and this only occurs in the presence of caveolin-1. The mechanism of how this transport may occur is still unknown. One possibility would be that endosomal-engulfed NORC may move through the cell along a microtubule cytoskeletal network.

Endocytosis & Autosis

The occurrence of NKA regulated cell death via non-apoptotic processes is identified as ‘autosis’. This process is caused by hypoxia-ischemia, autophagy-inducing peptides, and starvation which is often recognized within cells by reduction of the endoplasmic reticulum and swelling between the nuclear membrane (Yang et al., 2015). Autosis via lysosomal degradation, provides eukaryotic cells with ways of adapting to stressors within their surrounding environments, which prove pivotal for cell survival. (Liu et al., 2013). The mechanisms involved in autosis, however, have yet to be elucidated, one possibility could involve endogenous ouabain as a mediator. “Cardiac glycosides prevent autophagic cell death by autosis instigated by starvation. (Liu et al., 2015). This protective effect has also been seen in vivo: during cerebral hypoxia or ischemia – endogenous ouabain or endobain is released from brain inhibiting in an autorcrine manner NKA (Yang et al., 2013). Neonatal brain tissues release of cardiac glycoside in cases of hypoxia-ischemia, suggest an evolutionary mechanisms related to the preservation of cell viability via autosis reduction (Yang, 2013). With the enlistment of caveolin-1 and the protective
effects of ouabain along with other endogenous cardiac glycosides, NORC may associate with pro- and anti-apoptotic proteins not restricted to the cell membrane.

**Tubulin and Microtubules**

The transit of the proposed NORC from the membrane to and through the cytoplasm constitutes another unknown factor. One explanation could be the transport along a known system of microtubules (MT). MTs are non-static tubular polymers consisting of α/β-tubulin dimers. These dimers interact to form cytoplasmic protofilaments which transport intracellular materials, and provide structural arrangement for cytoplasmic contents in eukaryotic cells (Al-Bassam, 2012). MTs are formed within cells via the polymerization of the α/β-tubulin units creating a cylindrical structure (Figure 1.3).

![Figure 1.3. Microtubule polymerization](image)

A graphic representation of the α/β-tubulin heterodimers and their interaction and polymerizations into MT structures.
MTs follow a process of polymerization and depolymerization activated by a c-terminally located tyrosine residue on MTs. Following the removal of this tyrosine via tyrosine carboxypeptidase, MT is depolymerized (Amaiden et al., 2015). Another type of tubulin modulation produces an acetylated α-tubulin subset for which structural modeling provided details surrounding the preservation of nearby tubulin protofilaments achieved by acetylation explaining increased stabilization observed in acetylated MTs (Al-Bassam et al., 2012). Tubulin is post translationally modified via addition of an acetyl group to the ε-amino group of Lys40 of α-tubulin. The stabilized form of MTs has been claimed to interact directly with NKA (Zampar et al., 2009). Therefore, NKA may function as a preliminary site for microtubules attachment to the plasma membrane, and vice versa. MT’s may also then, “grab” NKA before the latter enters the escalator into the deeper realms of the cell. It is the 5th cytoplasmic domain of NKA that has been proposed to interact with acetylated tubulin (Zampar et al., 2009).

**Steroid Receptor coactivator 1 & 3 (SRC 1 & SRC 3)**

A possible outcome of the internalization of NORC could be its lysosomal degradation, and separation of NKA and ouabain. Upon separating, the latter may interact with steroid nuclear receptors should its steroidal core move through the endoplasmic reticulum. This commensurate with similar binding found between the cardiac glycoside bufalin and SRC (Jin, O’Malley et al., 2014). Bufalin has been shown to reduce the expression of SRC-1 and SRC-3 nuclear receptor proteins. Reductions in expression when tested in vitro and in vivo, showed decreased proliferation of cancer cells (Zhou, 2005). SRC-3 plays a significant role in cell viability and proliferation. Consequent to declining SRC-3 levels, Bcl-2 and mRNA levels were also significantly reduced (Zhou, 2005). If holding true for other CTS, ouabain may interact similarly to bufalin explaining its positive
attenuation of some cancer types as well as the regulatory apoptotic mechanisms through the attenuation of Bcl-2 protein expression.

**Significance**

With a broad variety of CTS to NKA effects, it must also be determined if these mechanisms are functioning through an undefined process that involves NORC internalization. We therefore propose a “microtubule escalator” that may exist, or be polymerized, because of ouabain’s interaction with NKA. This transit network of MT-assisted internalization would occur by first inhibition of Na⁺/K⁺ exchange through assembly of ouabain and NKA. Indeed MT associated transport of other membrane transporters and receptors such. In response to ouabain inhibition, the caveolin-1 assisted NORC invagination through the plasmalemmal membrane occurs. This invagination is then followed by transport from early endosome to lysosomal vesicles within the cytosol. Lysosomal bodies could then break down NORC to NKA for recycling to the plasmalemmal membrane, and ouabain for processing in the endoplasmic reticulum. Once processed in the endoplasmic reticulum, ouabain may interact with nuclear receptors, causing subsequent gene upregulation. Figure 1.4 represents the current model and expected results from previous experiments of this project, and from the literature. Should experimentation echo similar action to the above, further identification of affected regulatory mechanisms would need be elucidated. We propose NKA’s and CTS’s intracellular actions may occur either as a complex (NORC) or by via cleavage of ouabain from NKA, where the former could “hormonally” modulate steroidal receptor coactivators at the nucleus. If these mechanisms are confirmed, their relevance for transcriptional modification need to be identified.
Figure 1.4. Preliminary model of anticipated NORC internalization

Proposed novel model of NORC internalization and subsequent breakdown of the complex via lysosomal degradation.
II. MATERIALS AND METHODS

Cell Culture

One tube containing human lens epithelial B-3 cells were removed from liquid nitrogen and thawed in a water bath. Then, 1 mL cells were added to a T75 vented flask and mixed with 17 mL of warmed culture medium (72 % Eagle Minimum Essential Medium, 18 % KGM Keratinocyte media, 5 % Horse Serum, 5 % Fetal clone III serum, and 2 mL gentamycin) and placed in an incubator at 37 °C 5 % CO₂. The medium was replaced the day after thawing and then every 2 d. Once cells reached 90 % confluence they were split into 3 new flasks. Media were first pre-warmed in a water bath (37 °C). The exhausted medium in the B-3 cell containing flask was removed and replaced with 3 mL PBS, then rocked to ensure even covering. PBS was aspirated and then replaced with 2 mL of 0.25 % trypsin and let to sit for 1 min to detach cells. Once cells are detached, they were resuspended with 7 mL of the pre-warmed media. The suspension was pipetted up and down ten times to homogenize the mixture without damaging cells. A 3 mL aliquot of the cell suspension was then added to new flasks and 15 mL of warmed media were added to each and placed in the incubator. Upon reaching 80 % confluence, 3 mL trypsin and 10 mL of the warmed B-3 media were added to re-suspend the cells in a flask. Cells were counted by adding 2 μl Trypan blue to 80 μl of the cell suspension. 10 μl of the Trypan blue/cell suspension mixture was added to a Neubauer chamber for cell counting. Afterwards, 5x10⁴ cells were added to each well of a sterile Millicell EZ SLIDE 8-well
glass and placed into the incubator until reaching 70% confluence and used for immunocytochemistry.

**Immunocytochemistry**

B-3 cells processed as indicated above, were washed 2X with 0.3 mL of K+ free phosphate buffer solution (K+PBS). K+PBS was used to prevent ouabain dissociation from NKA by K+. Cells were incubated with a variety of cold (unlabeled) ouabain, CO, or BODIPY fluorescent ouabain (BFO) concentrations, depending on the study (ranging from $10^{-9}$ μM to a maximum 5 mM) for 45 min. If exposed to BFO the remainder of the experiment was performed in a poorly lit room to prevent degradation of the fluorophores by photo bleaching. B-3 cells were fixed using a 4% paraformaldehyde, with 0.1% saponin (PFA), either before or after incubations with ouabain concentrations, dependent on the study. After ouabain incubation or PFA fixation, cells were washed 2X with 0.3 mL K+PBS. Blocking of cells was done with 3% normal donkey serum (NDS) in K+PBS. Blocked cells were incubated with primary antibodies in 3% NDS against specific proteins dependent on study, overnight (Bcl-XL, Caveolin-1, Acetylated Tubulin, α Tubulin, Actin, α1-subunit NKA, or β1-subunit NKA). The preceding day or, 18 h later, cells were washed 3X with K+PBS. Cells were incubated using Alexa-Fluor-conjugated (green) donkey anti-rabbit and/or Cy3 conjugated (red) donkey anti-mouse secondary antibodies to label their respective proteins. Next, chambers were washed once with distilled water before removing the chamber separator. Lastly a few drops of Vectashield (antifade solution) were placed over the cells and a coverslip was mounted on the slide. After placing the mounting cover slip, the slides were prepared for imaging.

**Epifluorescent Imaging and Colocalization**
Images were collected using a Nikon Eclipse E400 epifluorescent Microscope under DAPI, FITC, and TRITC filters detecting blue, green, and red emissions, respectively. Figures were collected at the 100X magnification, and edited using Gimp2 software. Edits in Gimp 2 dimmed emissions from the antibodies to selectively identify structures. Images were overlaid and merged using Adobe Photoshop. Merged images were used to identify regions of possible interaction through colocalization, determined by orange or yellow overlapping sections.

**Protein Extraction**

B-3 cells were split and 1x10⁶ cells were added to each of 8 tissue culture plates with media. Plates were incubated (37 °C, 5 % CO₂) for 5 d to reach 90 % confluence. Media were aspirated before adding 5 mL PBS and gently swirling to wash cells followed by aspirating the solution. A solution of HALT mixed with 1x PBS (PBS-HT) at (dilution 1:100) was prepared, and 2 mL added to all plates. Plates with PBS-HT were placed on ice for 5 min. All cells were scraped off the tissue culture plates using a cell-scaper and decanted into 2 mL centrifuge tubes adding approximately 1.5 mL of the suspension. Tubes were centrifuged at 13,500 RPM 4 °C for 10 min to create a pellet. 150 µl of RIPA was pipetted up and down into each centrifuge tube resuspending the pellet and lysing cells. Next, tubes were incubated on ice for 30 min, and vortexed every 6 min. All tubes were sonicated (setting 6) for 6 min each before centrifuging at 13,500 RPM 4 °C for 10 min, and stored in a -80 °C refrigerator until needed for Western Blot or RT-OCR.

**Western Blots**

Protein extracts were collected and thawed in a water bath. A solution of 40 mM Tris Base, 20 mM sodium acetate, 2 mM sodium ethylene diamine tetra acetic acid
(EDTA), and 0.2 % sodium dodecyl sulfate (SDS) (tank buffer) was filled into the electrophoresis chamber. The outside of the gel plates was washed with deionized H2O to prevent contamination. Next 25 µL of protein extract treated with LaemmLi buffer was added to each well. The first well was loaded with 15 µL of protein ladder (Thermo Scientific, Spectra Multicolor Broad Range Protein Ladder, Lot#00410568) added for reference. Gel electrophoresis was performed at 150 V and 150 mA for 90 min until the protein ladder reached the bottom of the gel. Gels were then electro-transferred to a PVDF membrane at 30 V and 90 mA for 18 h. Upon completion of the transfer, the iBind Western Systems apparatus (Thermo Scientific) was used to stain the protein bands on the PVDF membrane with their respective primary and secondary antibodies. Thereafter, the membrane was rinsed in Super Signal West Pico Plus (Thermo Scientific, Lot# RK239827) for 5 min and images were collected at high sensitivity and high resolution using ImageLab 4.1 software.
III. RESULTS

Experimental design

The predictions of the model detailed in Figure 1.4, were tested in immortalized human lens epithelial cells B-3 cells. These cells were chosen as this laboratory had characterized some of its membrane ion transporters, including NKA in the past (Lauf et al., 2006). NKA, RT-PCR was performed to identify the isoforms of α and β subunits by Professor Gustavo Blanco (see acknowledgements). Proteins presumably participating in the NORC internalization process were identified by Western blot with the help of Gousia Begum. The presence of NKA binding site for these proteins was predicted from a MAFFT-based peptide sequence analysis. Once the proteins had been confirmed, Immunocolocalization studies were done to identify their colocalization with NORC as denoted by yellow/orange coloration from overlaid epifluorescence images.

**B-3 cells contain α1 and β1-3 subunits**

RNAlater-stabilized B-3 cell extracts were shipped to Professor Gustavo Blanco to determine α and β NKA subunits by RTPCR. Figure 3.1 shows control brain sample presence of cDNA for α1, 2 and 3 (α4 was omitted as only present in sperm, see Blanco and Mercer, 1998) and for β1, and 3, but in B-3 cells only α1, and β1 and β3, henceforth conforming with the general notion that epithelial cells possess preferentially these isoforms. Other controls such as GAPDH and water were as expected. As a functional NKA has both α1 and β1 subunits their identification in B-3 cells is important to determine the efficacy of B-3 cells as a model. However, it should be pointed out, that earlier work
from this laboratory showed sizable ouabain-sensitive Na/K pump fluxes (Lauf et al., 2006), commensurate with the presence of fully active NKA αβ dimers in B-3 cells.

**Figure 3.1. RT-PCR to detect HLE-B-3 RNA expression of NKA subunits**

Results from RT-PCR of B-3 cell line to determine subunit expression of NKA. B-3 cells were used as candidate sample with brain as positive and deionized water as negative controls. GAPDH in B-3 cells, brain, testis, and water served as internal standard. Results showed identification of α1, β1, and β3 NKA subunit expression in B-3 cells.

**MAFFT based analysis of NKA α1 subunit protein interactions**

MAFFT-based analysis was performed to display published available binding sites for proteins of interest from the amino acid sequence of NKA as shown in Figure 3.2. This information matched with the results from the literature to confirm that the proteins used in the Figure 1.4 model were plausible binding partners to NORC. From the results gathered, notable regions of interest occurred as expected for Bcl-2 BH-1 and BH-3 motifs. For membrane invagination of the receptor and subsequent transport of NORC there were
also 2 binding sites for Caveolin-1 as well as the acetylated tubulin (as proposed by Zampar et al., 2009). Cherniavsky-Lev et al., (2014) had previously shown colocalization of bioengineered YPF-tagged NKA with the lysosomal marker LAMP1. Hence, we added as an intermediate step the early endosomal marker EEA1 to probe for its colocalization with NKA. Note the absence of EEA1 and LAMP1 interacting amino acid sequences in Figure 3.2, as to our knowledge this information is missing.

**MAFFT-BASED ANALYSIS OF 10 Na/K ATPase (Sus scrofa)–PROTEIN INTERACTION SITES.**

![MAFFT-BASED ANALYSIS OF 10 Na/K ATPase (Sus scrofa)–PROTEIN INTERACTION SITES.](image)

Figure 3.2. MAFFT based analysis of NKA of potential protein binding motifs

MAFFT-based analysis showing likely protein binding site sequences on α1-NKA as highlighted by the following colors: Yellow for BH-1 and grey for BH-3 motifs, light blue for caveolin-1, and grey for Oac-tubulin. Other, indeed interesting sites, possibly participating in the formation of a complete NORC, such as binding of coflin, PI3K (phosphoinositol-3 protein kinase), IP3R (inositol phosphate-3 receptor), ankyrin and AP2TF (apoptosis-2 transcription factor) are also indicated but were not further pursued in this thesis.
HLE-B-3 cells express $\alpha_1$-NKA, Caveolin-1, Bcl-XL (XS), $\alpha$-tubulin, EEA1, LAMP1 and $\beta$-Actin, but not the $\beta_1$-NKA subunit.

To affirm that proteins for interaction with NKA were present within B-3 cells, Western blots were performed with the assistance of Gousia Begum, Year 1 P&T Graduate Student. Electrophoresis staining with respective antibodies for $\alpha_1$-NKA, caveolin-1, Bcl-XL, $\alpha$-tubulin, EEA1, LAMP1 and $\beta$-Actin were positively detected. The $\beta_1$-NKA subunit, however, was absent in its respective band (Figure 3.3). While gene transcription occurred as seen by RT-PCR (Figure 3.1), the gene product apparently is not translated at the protein level. Whether the $\alpha_1$ subunit prefers “the brunette” $\beta_3$ over “the blonde” $\beta_1$ subunit to complement the fully functional NKA, is currently under investigation.

Interestingly, another oddity is apparent in that the Bcl-XL protein molecular mass was closer to 19 kDa range than to its anticipated 27 kDa. Indeed, the manufacturing data sheet details a possible detection by the anti-BclXL antibody of two variants, Bcl-XL and Bcl-XS. In previous work we showed presence of the full size pro-survival BclXL in FHL124 cells (Lauf et al., 2015) but no evidence for BclXS. B-3 cells do not translate the full peptide and instead display Bcl-XS, a truncated form of Bcl-XL protein with pro-apoptotic action vs. the anti-apoptotic effects of the latter. This change in molecular mass is due to the missing BH-1 groove sequence that would normally interact with BH-3 motifs of pro-apoptotic peptides within cells. These two molecular deviations may be a characteristic of the B-3 cell line which originally was SV40-immortalized (Andley et al., 1994). A third molecular oddity, the absence of a specific protein kinase isoform, is still under investigation in this laboratory.
Western blots of B-3 protein extracts to determine that proteins of interest (\(\alpha_1\)-NKA, \(\beta_1\)-NKA, \(\alpha\)-tubulin, Bcl-XL, caveolin-1, EEA1, and Lamp1) were post-transcriptionally translated and consistent with the model. \(\beta\)-actin was a positive control.

**Ouabain binds exclusively to the NKA Receptor**

As the \(\alpha_1\)-subunit was found in B-3 cell extracts, binding of Bodipy-fluorescent ouabain (BFO) was confirmed. Cells were incubated and live-stained with both 2 \(\mu\)M BFO for 45 min and, after fixation with paraformaldehyde, with an AB specific for the \(\alpha_1\)-subunit of the NKA receptor. Figure 3.4 revealed faint live green-staining by BFO alone, and Cy3-red staining of NKA with an orange overlap of the receptor-ligand complex only with minimal unbound BFO (green). This interaction echoes previous studies of the selective binding of CTS compounds exclusively to the NKA receptors. Note the significant colocalization identified within the cytoplasm. During this experiment, concentrations ranging from 0 \(\mu\)M - 5 \(\mu\)M BFO were used with B-3 cells. Hence this experiment shows beyond doubt that NORC, which we call the minimum complex between NKA receptor and ouabain, enters the cytoplasm and thus unifies previous observation of
individual cytosolic appearances of BFO (Alonso et al., 2013) and of bioengineered YFP-tagged NKA (Cherniavsky-Lev et al., 2014). Interestingly, differing cell sizes were noted as BFO concentrations increased (not shown here). Therefore, it was decided to study the effect of varying unlabeled (cold) ouabain on B-3 cell size.

**Figure 3.4 Immunocolocalization of NKA/BODIPY fluorescent ouabain**

B-3 cells were exposed live for 45 min 2.5 µM BODIPY green-fluorescent ouabain (BFO) and subsequently with Cy3 red fluorescence-labeled secondary AB to primary AB against the α-1 subunit of NKA. The structural/topographic closeness of the chemical ouabain with the NKA receptor became evident through orange/yellow fluorescence revealing colocalization in merged images.
Cell size increases as a function of ouabain concentration

As stated in the preceding paragraph, increasing BFO concentrations caused larger cell sizes. Thus cells were incubated in differing unlabeled or cold ouabain (CO) concentrations per chamber well to assess their effect on cell size. As concentrations were elevated, increased cell sizes were observed (Figure 3.5): At 2.5 µM ouabain, cell size increased by an approximate 10 %, at 4 µM about by 65 %, and by 110 % at 5 µM. The expanding cell size may be attributed to increase in water retention as CG inhibition of NKA is known to increase Na⁺ content of the cytosol. Cells are known to retain water to regulate their tonicity by a variety of membrane transport mechanisms, a process coined volume regulation (Hoffmann et al., 1989). Alternatively, previous studies have shown possible interactions with NKA and Bcl-2 proteins such as Bcl-XL (Lauf, 2015). In response to stressors such as ouabain, associated cell size increase may proceed apoptosis or necrosis.
Localization of α1-subunit of NKA (red) antibodies at different CO concentrations (µM) per chamber well: 0, 2.5, 4.0, and 5.0 show variations in cell size.

**Bcl-XL and NKA colocalize independently of CO concentration**

Commensurate with studies proposing anti-apoptotic mechanism induced by interactions with NKA and Bcl-2 proteins (Lauf et al., 2015), antibodies against Bcl-XL and NKA were added to B-3 cell cultures. Varying concentrations of CO were used to see whether Bcl-2 colocalization with NKA was affected by the ouabain concentration. Increasing by tenfold concentration steps from 1 nM to 5 mM revealed colocalizations as seen by the yellow overlaps (Figure 3.6). Colocalization was not affected by cellular exposure to increasing concentrations of CO and occurred in some cells. There was also no
distinct pattern in Bcl-XL colocalization with NKA. It should be pointed out that the immunochemical pictures are showing colocalization of BclXS with NKA, as discussed earlier, which was independent of ouabain concentrations. This finding is interesting, as it tells us that BclXL and BclXS may colocalize with NKA via the same mechanism in FHL124 cells (Lauf et al., 2015), and B-3 cells (this study), respectively, utilizing the BH-1-like motif in the N-terminus of NKA to putatively attract the BH-3 motif of the BH-1 motif-devoid BclXS. Further studies are needed to shed additional light on this phenomenon.
Figure 3.6 Immunocolocalization of NKA/Bcl-XS

Colocalization of Anti-Bcl-XL (green) antibodies with α1-subunit of NKA (red) antibodies at varying concentrations of CO (µM): (A) 1x10⁻⁹, (B) 1x10⁻⁷, (C) 1x10⁻⁶, (D) 1x10⁻⁵, (E) 1x10⁻⁴, (F) 5x 10⁻³. Colocalization was identified by overlap appearing yellow or orange in merged images (blue arrows). The data likely characterize the NKA-BclXS interplay in terms of a putative interaction between the NKA’s BH-1 motif and BclXS BH-3 motif.

NKA colocalized with caveolin-1 independent of the presence of ouabain

A large body of evidence primarily by the Xie research group (Xie, Dong, 2011, Xie, et al., 2004, Xie, et al., 2008) suggests that caveolin interacts with NKA in the presence and absence of ouabain in LLC-PK1 cells (Liu et al., 2004). To ensure similar interactions occurred in B-3 cells, caveolin-1 was tested for colocalization with NKA in the presence and absence of 250 µM CO. The experiment in Figure 3.7 demonstrated comparable colocalization by yellow overlap in both circumstances, i.e. ouabain did not affect the interaction of NORC with caveolin-1. Nevertheless, caveolin was used as a positive control for NKA colocalization studies and provided a conceivable means to detect endocytosis of NKA. As transport mechanisms of NORC are still unknown, additional studies are required to elucidate further this aspect of NORC internalization.
Figure 3.7 Immunocolocalization of NKA/Caveolin-1

Immuno-histochemical staining of caveolin-1 and α1-subunit of NKA by their respective primary antibodies followed by green and red secondary antibodies, with and without 250 µM CO. Colocalization was confirmed by yellow or orange overlap in merged images (blue arrows).

NKA interacts with α-tubulin in the presence of ouabain.

It has been reported that tubulin, especially in its acetylated form interacts with the 5th cytoplasmic domain NKA (Zampar et al., 2009). This observation has not yet been repeated. Therefore, we attempted to verify microtubules as an essential component of the cytosolic escalator for NORC. Thus, B-3 cells were incubated with or without 250 µM CO while simultaneously labeling the α1-subunit of NKA (red) and α-tubulin (green). Figure 3.8 shows the typical microtubule filaments in the absence of ouabain, and no apparent interaction between α-tubulin and the α1 subunit of NKA. This finding is clearly at variance with the report by Zampar et al. (2009). However, when B-3 cells were treated with 250 µM CO, the filamentous structure of microtubules disappeared and dots of colocalization between NKA and microtubules were seen in the lower region of the cytoplasm as well as left of the nucleus (Figure 3.8), a finding not reported before. Another colocalization
experiment was then performed at CO concentrations, from 0 to 1.0 µM (Figure 3.9). Overlap between NKA and α-tubulin was recognized based on yellow in the merged images of Figure 3. Increases in CO concentration correlated with greater levels of colocalization between NKA and α-tubulin. This result is principally commensurate with that of Zampar et al. (2009), however, no observed NKA-tubulin interaction occurred in the absence of ouabain.

Figure 3.8 Immunocolocalization of NKA/α-Tubulin

Immuno-histochemical staining of α-tubulin and α-1 subunit of NKA with their respective primary and secondary green and red fluorescent antibodies, with and without ouabain 250 µM CO. Colocalization confirmed as yellow and orange overlapping images (blue arrows).

In addition, Zamper et al. (2009) showed that for interaction with NKA tubulin needed be Lys40 O-acetylated by inhibiting deacetylation of microtubules with Tricostatin (TSA). The MAFFT-analysis in Figure 3.2 considers the proposed NKA binding site for acetylated MT (tubulin) comprising a 934-950 at the C-terminus of the molecule. As initially anticipated, NKA colocalized with α-tubulin in significantly more locations throughout the cell after incubation with the deacetylase inhibitor, TSA (Figure 3.10).
Figure 3.9 Immunocolocalization of NKA/α-Tubulin at variable ouabain concentrations

Immuno-histochemical staining of anti-α-tubulin and anti-α1-NKA subunit with respective primary and red/green secondary antibodies at increasing ouabain concentrations (µM) (1) 0, (2) 0.01, (3) 0.1, and (4) 1.0. Colocalization was improved with increased CO concentration as evident in the yellow and orange overlapping images (blue arrows).

Pretreatment of B-3 cells with 1 µM TSA indeed led to colocalization of NKA with MTs as shown in Figure 3.10, left tetramer. However, unexpectedly, when 1µM ouabain was present, the colocalization signal completely vanished. Figure 3.10, right tetramer.
To summarize the findings with NKA and tubulin, with and without ouabain and TSA. First, NKA and tubulin showed no interaction with unmodified microtubules, but increasing CO concentrations clearly revealed colocalization. Second, NKA colocalized with Oac− tubulin, in TSA-treated cells, commensurate with the earlier report of Zampar et al. 2009. However, when 1 µM ouabain was present, colocalization was attenuated. Hence ouabain and TSA acted antagonistically in terms of the colocalization of NKA with MTs. A mechanistic explanation may be offered in the Discussion section.

![Figure 3.10 Immunocolocalization of NKA/α-Tubulin/deacetylase inhibitor (TSA)](image)

**Figure 3.10 Immunocolocalization of NKA/α-Tubulin/deacetylase inhibitor (TSA)**

Immuno-histochemical staining of anti-α-tubulin and anti-α1-NKA subunit with respective primary and red/green secondary antibodies, at zero and 1 µM ouabain and in the presence of 1 µM Tricostatin-A. Colocalization confirmed as yellow and orange overlapping images (indicated by blue arrows).

**Early endosomal interactions with NKA are not affected by exposure to increasing ouabain concentration.**

Increasing CO concentrations was without effect on colocalization of NKA and early endosomes. In almost all instances colocalization was seen between NKA and EEA1 (Figure 3.11). The absence of ouabain did not abolish the endosomal overlay with NKA as anticipated, however. This means that the NKA-EEA1 pairs formed without CO do
represent NKA cycling through the endosomes perhaps on the way back to the plasma membrane. It would be imperative to find out whether NKA with ouabain, i.e. NORC, does the same or move straight to the lysosomes.

Figure 3.11 Immunocolocalization of NKA/EEA1

Immuno-histochemical staining of anti-EEA1 and α-1 subunit of NKA with respective Primary and red-/green secondary antibodies, with increasing ouabain concentrations (µM) (1) 0.0, (2) 0.01, (3) 0.1, and (4) 1.0. Colocalization of NKA and EEA1 confirmed as yellow and orange overlapping images (Identified by blue arrows).
**Increased ouabain concentration effects lysosomal interactions with NORC**

Bioengineered YFP-NKA and LAMP 1 colocalized in lysosomes as shown by Cherniavsky-Lev et al. (2014). When testing the colocalization of NKA against LAMP1 (Figure 3.12) there was markedly more colocalization in cells exposed to higher ouabain concentrations commensurate with the Cherniavsky-Lev et al. (2014) data. If one assumes that more NORC is formed with more ouabain around, one may conclude that NORC more likely reaches the lysosomes for subsequent hydrolysis of the complex and further signaling. Certainly, experiments with BFO would be decisive for a final answer.

![Figure 3.12 Immunocolocalization of NKA/LAMP1](image)

**Figure 3.12 Immunocolocalization of NKA/LAMP1**

Immuno-histochemical staining of anti-LAMP1 and α-1 subunit of NKA with respective primary and red/green secondary antibodies, with increasing ouabain concentrations.
(µM) (1) 0, (2) 0.01, (3) 0.1, and (4) 1.0. Colocalization confirmed as yellow and orange overlapping images (identified by blue arrows).
IV. DISCUSSIONS AND CONCLUSION

The present work addresses still unknown details of the cytosolic internalization of what it is called here, NORC, which is an acronym for the binding and complex (C) formation of CTS, particularly of ouabain (O), with the NKA (N) receptor (R). Although not defined as in this work, NORC formation without its internalization has been known for more than seven decades to precede the principal and first pharmacological action of CTS to cause inotropy by inverting the Na/K gradients and stimulating reversal of the Na/Calcium exchanger leading to increasing cytosolic Ca ions and strengthening of muscle contraction (modalityskou A) (Nishio et al., 2002, Mohammadi et al., 2003). During the last 3 decades, NORC formation without internalization was recognized to elicit signal cascades comprised of tyrosine (Y) kinases starting with the SRC tyrosine kinase (Modality B) (Cui and Xie, 2017). Subsequent ERK1/2 and MAPK activation are followed by downstream transcriptional effects. Activation of PI3K (Modality C) with subsequent mobilization of cytosolic Ca ions through the IP3R which in turn augment mitochondrial metabolism of ATP and ROS formation, again effect transcriptional upregulation of genes important for cellular survival (Liu et al., 2004, Aperia et al., 2016). However, that NORC, by cytosolic internalization (modality D), would constitute another mechanism of cell signaling, after its endosomal and lysosomal processing, is of much more recent interest, although several publications have suggested this possibility (Aydemir-Koksoy et al., 2001).
As it is always in science, advances in technology facilitated to study above listed 4 modalities, consecutively. Thus, in modality A, it was the detection of Na + K-dependent ATP hydrolysis by the ouabain-sensitive NKA (Skou et al., 1992, Post R.L. et al., 1972), cytosolic ionic changes by atomic absorption spectroscopy (Habermann, Chhatwal, 1981), and the tritium labeling of ouabain to assess the NKA pump turnover in living cells (Lauf and Joiner 1978 a, b), all of which were instrumental techniques. In modality B and C, molecular biology and protein techniques enabled detection of SRC and PI3K association with NKA and the consequent gene upregulation (Nissen et al., 2012, Pedemonte et al., 2005). Modality D, neglected for a while since seen first by immunocytochemistry, required additional special tools such as BODIPY labeling of ouabain, the availability of confocal microscopy and Förster resonance energy transfer (FRET) analysis (Sekar et al., 2003).

One of the first questions to be solved was that BODIPY fluorescent ouabain (BFO) was transferred as part of NORC and not simply due to diffusion of the lipophilic compound, either binding as contamination to the plasma membrane or after a glycosidase-mediated hydrolysis of the chromophore from ouabain. BFO was detected as part of NORC within the membrane and cytosol by immune-colocalization of live-staining with the green fluorescent BFO and the red fluorophore CY3 on the secondary antibody against the NKA specific antibody, at least 45 min after incubation of B-3 cells with BFO and successive immunochemical red staining as shown in Figure 3.3. Thus, our data resolved the question arising from the work of Alonso et al 2013: BFO is part of NORC that reaches the cytosol. An independent approach to demonstrate cytosolic NORC was provided by dark field amplified microscopy with a CytoViva equipment (not shown), but this study did not use
secondary immune-staining with anti-α1 NKA antibodies. However, work on this aspect is in progress.

Several proteins known or proposed, in part based on sequence alignments, to interact with NKA were caveolin-1 (Dong et al., 2011, Yosef et al., 2016, Chakraborti et al., 2015), BclXL (Lauf et al., 2015, Aperia et al., 2016), tubulin, early endosomal antigen (EEA1) and LAMP-1, the lysosomal associated membrane protein 1.

Western Blot analysis showed the presence of these proteins (Figure 3.2, Figure 3.3). Furthermore, B-3 cells have only the α1 catalytic subunit (Figure 3.3) since the genes for the α2 and α3 NKA subunits were absent (Figure 3.1). Thus, B-3 cells are typical epithelial cells possessing, always and only, the α1 subunit which in all studies published thus far, is tightly linked with the CTS-activated src-EGFR-ERK1/2 signaling pathway (Cui and Xie, 2017). The α1 subunit is generally associated with the β1 glycosylated subunit. However, although transcribed, the Western Blot (Figure 3.3) failed to detect this protein. Whether instead, the α1 associates with a β3 subunit is possible, however, this interaction has not been further elucidated in this work and is currently under investigation.

The B-3 cell line is an SV40-immortallized cell line, where the p53 levels are low or absent (Andley et al., 1994) and thus apoptosis is minimized, also a hallmark of tumor cells. It is therefore interesting that B-3 cells lack the 27 kDa BclXL with its full set of BH-1, -2, -3, and -4 motifs, and instead, possess the smaller 19 kDa molecular weight version of BclXL/S that lack the BH1 motif (Chang et al., 1999), and therefore become a pro-apoptotic protein carrying the reactive BH-3 hair loop pin that is binding and neutralizing the fully pro-survival proteins Bcl-2 and BclXL. Since the NKA α1 subunit has been proposed to contain a BH1-like sequence of 13 amino acids in its N-terminus, there should
be binding of BclXS BH-3 to the former. Indeed, as Figure 3.5 showed, there was clear colocalization between the CY3-red stained α1 NKA subunit and the green fluorescence of anti-BclXL. Since in B-3 cells, the classic protein mass staining of 27 kDa is replaced by that of a 19 kDa protein, one must assume that both BclXL and BclXS share a common antigenic epitope against which anti-BclXL is directed. Given the fact that BclXS is a BH-3 motif only containing peptide, the data suggest that the BclXL and hence BclXS interactions must occur within the shallow BH1-like domain of the N-terminus of NKA (a.a. 59-71, see Lauf et al., 2013, and Lauf et al., 2015). It is interesting that the putative immune colocalization between NKA’s N-terminal BH-1 like motif and BclXS’ BH-3 motif is unaffected by the presence of unlabeled ouabain from $10^{-9}$ to $5 \times 10^{-3}$ M. The N terminal domain rotates by $120^\circ$ during the catalytic cycle of NKA to protect the asp (D) 369 ATP-mediated phosphorylation and K-dependent dephosphorylation by in line hydrolysis. The data indicate that the NKAS-BclXL(s) protein-protein interaction may not affect the canonical cycle of NKA. However, this conclusion needs to be functionally tested through ion fluxes in cultures where all cells show this NKA-BclXL(s) interaction. Indeed, chelerythrine, a BH-3 mimetic, inhibits the NKA pump fluxes (Lauf et al., 2013), perhaps by a mechanism proposed here for BclXL(s) that may affect the transit of 2KE2 to 3NaE1–P in the canonical model.

Bcl-2 proteins affect the kinetics of α-tubulin polymerization (Knipling & Wolf, 2006), and MT formation has been linked to NKA because its 16-amino acid long peptide in the C-terminal residues 934-960 interacts with tubulin in the presence of Tricostatin, an inhibitor of protein deacetylases, i.e. when tubulin is acetylated (Zampar et al., 2009). Thus, it was only logical to postulate that part of the intracellular escalator mechanism
transporting NKA or NORC into endosomes and lysosomes might be acetylated tubulin. Figure 3.8 shows that indeed there was immune histochemical evidence for colocalization of NKA with α tubulin in some cells but not in others. This effect was amplified when the external ouabain concentrations was raised to 1 µM. However, in the presence of Tricostatin and ouabain there was no α-tubulin-NKA interaction. This data suggests that it may be the tubulin/NKA protein/protein interaction that is ouabain-sensitive and accelerated, rather than its acetylated form as proposed by Zampa et al. (2009). It should be also noted here, that exposure of B-3 cells to ouabain alone increased their cell size (Figure 3.4) commensurate with Na and water entry into the cells and possibly α tubulin depolymerization. It is unknown if such a process drives tubulin-NKA interaction and redistribution as observed in this work (Figure.3.3, and Figure 3.8). There is evidence that MTs extend from the plasmalemma to mitochondria. Although not tested for tubulin, Alonso et al., (2013) surmised from their BFO internalization data that, if proven to be bound to NKA, the latter would associate in the cytosol close to mitochondria.

Work from Xie’s group has shown that NKA associates with caveolin-1 (Liu et al. 2005). We confirmed this finding (Figure 3.6). Unless caveolin-1 participates through tubulin in the cytosolic escalator mechanism, our finding thus constitutes mainly a control experiment to demonstrate colocalization. However, the sub-plasmalemmal localization of NORC or NKA with tubulin suggests that the latter provides the scaffold for the descent of either into the endo- and lysosomal organelles. Our data that NKA colocalizes with EEA1 and LAMP1 are commensurate with the finding by Karlish and collaborators (2014) that a YFP-tagged NKA bio-construct could be traced to early and late endosomes, and to lysosomes. Interestingly, inconsistent with conventional knowledge, endosomes in B-3
cells must be positioned below the plasma membrane as the yellow/orange overlap fluorescence indicated.

**Conclusion**

Our studies have established: 1). B-3 cells possess the α1NKA subunit. Whether it associates to a functioning unit with β1 or β3 is still to be shown. Subsequently to binding of ouabain to the α1-NKA receptor, the minimally binary complex NORC is translocated across the plasma membrane as BFO and antibody labeled NKA colocalized into the cell’s cytosol. 2). Putatively, NORC interacts with BclXL/S, and with (non-acetylated) tubulin in an ouabain concentration-dependent manner. 3). Microtubule (MT) acetylation (by means of TSA inhibition of the deacetylase) appears to interfere with colocalization of NKA and MT in the presence of ouabain. Data are not yet available whether this quaternary complex might contain also caveolin-1. 4). Although we have not established the time course of events, and other details are missing, it appears plausible that the NKA without ouabain reaches the endosomes, as overlap was seen with EEA1. 5). Whether or not, NORC does the same for recycling purpose remains to be seen, more likely NORC associates with late endosomes and lysosomes, because its colocalization with LAMP1 was ouabain concentration-dependent. At this point, no further biochemical data are available to further substantiate these claims and to surmise NORC may be broken down to its unitary components. It is projected that ouabain most likely exits in its de-glycosylated form from these organelles and finds its way to nuclear receptor proteins such as SRC1 and 3 (Wang, O’Malley et al., 2014). Ouabain’s interaction with nuclear receptors would then exert its transcriptional upregulation of NKA as well as pro- and anti-apoptotic proteins. Work to support this projection is in progress. Figure 4.1 is an
attempt to generate a simplistic model of the cell biology of NORC’s descent into the cytosol presented in this work.
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