Are mitochondria a potential target for anti-cancer therapy in carcinoid tumors?

Shadi Zahedi
Medical University of Ohio

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Are Mitochondria a Potential Target for Anti-Cancer Therapy in Carcinoid Tumors?

by

Shadi Zahedi

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in Biomedical Sciences

Dr. David Giovannucci, Major Advisor

Dr. Andrew Beavis, Committee Member

Dr. Guillermo Vazquez, Committee Member

Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

August 2010
An Abstract of

Are Mitochondria a Potential Target for Anti-Cancer Therapy in Carcinoid Tumors?

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Gastrointestinal (GI) carcinoids are slow growing malignancies of neuroendocrine phenotype that can behave aggressively. To date, there are no effective therapies for metastatic carcinoid cancer. Previous work by our lab and others has shown that carcinoids express variety of voltage-operated (VOCCs) and non-voltage-operated Ca\(^{2+}\) channels to allow Ca\(^{2+}\) to enter the cell. Although, the role of Ca\(^{2+}\) entry in these tumors is not well understood, previous work by our group and others has shown that mitochondria are important regulators of voltage-operated and non-voltage-operated Ca\(^{2+}\) entry. In addition, cancer cells typically exhibit mitochondrial dysfunction and poor anti-oxidant status. These observations and the central role that mitochondria play in metabolism, Ca\(^{2+}\) homeostasis and cell death pathways make mitochondria an appealing potential target for anti-cancer treatment in carcinoid tumors. We used an spectrum of human cancer cell lines and a variety of microfluorescence methods including wide-field, confocal, and total internal reflection (TIRF) microscopy to assess Ca\(^{2+}\) signaling and mitochondrial function in combination with pharmacological interventions to assay whether mitochondria are a
potential target for anti-cancer therapy. To this end, we tested the effectiveness of an oxidant therapy approach in carcinoid cells.
To my father and mother who nourished my heart with love and infused me with the passion for knowledge.

To all those who strive beyond the ordinary and make life a more joyful experience for themselves and others.
Acknowledgements

I would like to express my most sincere thanks to Dr. David Giovannucci, my major advisor, for his exceptional guidance, invaluable advice, and continued support throughout my research. He provided me the opportunity to work and develop my skills in his laboratory and inspired me to strive for better every day and I would particularly like to acknowledge his belief in me, without which this work would not have been possible. I also would like to thank my committee members: Dr. Andrew Beavis, Dr. Guillermo Vazquez, and Dr. Zi-Jian Xie for their valuable guidance and suggestions as well as Dr. Sandrine Pierre for being the graduate school representative at my defense.

I would specially like to thank Dr. Christian G. Peters, Dr. Christine Brink, Dr. Tatiana Zhelay, Dr. Sasi Arunachalam, Jennifer Warner and all members of the Giovannucci laboratory for their kindness and support.

I am particularly grateful to Dr. Sonia M. Najjar and Dr. Keith Schlender for giving me the opportunity to experience the joy and challenges of life as a graduate student. Many thanks go to Dr. Roy Collaco and Dr. Joyce Bevington who helped and guided me through the preparation of my thesis presentation.

I am very fortunate to have Dr. Golleetah Alam as a good friend who helped me from the very early stages of my research and made this experience a more fulfilling one. I would also like to thank all my friends, family members, and those who supported me through this remarkable journey. Last but not least, I would like to thank my boyfriend...
Adam, for his unconditional love and support and for making this experience all the more memorable.
Table of Contents

Abstract iii
Contents viii
List of Figures ix
List of Abbreviations xxvi
Chapter 1: Introduction (Literature Review) 1
Chapter 2: Results 30
Chapter 3: Discussion 40
Chapter 4: Conclusion 46
Chapter 5: Materials & Methods 48
Chapter 6: References 51
List of Figures:

**Figure 1-1:** The InsP$_3$R Ca$^{2+}$ release channel. Cartoon depicting three of four InsP$_3$R molecules (in different colors) in a single tetrameric channel structure. Part of the luminal loop connecting transmembrane helices 5 and 6 of each monomer dips into the fourfold symmetrical axis, creating the permeation pathway for Ca$^{2+}$ efflux from the lumen of the endoplasmic reticulum. Adapted from Foskett et al. 2007
**Figure 1-2:** Domains identified in the ryanodine receptors (RyR) structure. (a) Schematic representation of the overall structure of the RyR, showing the relative localization of calmodulin (*purple*), Apo-calmodulin, calstabin (*blue*), the three divergent regions (*green*), the central disease-associated mutation region (*yellow*), and the pore region helices. (Zalk, Lehnart, & Marks, 2007)

**Figure 1-3:** cADPR and NAADP synthesis by ADP-ribosyl cyclase. NAADP and cADPR are synthesized by the same enzyme, ADP-ribosyl cyclase. The differential production of each depends on the pH. (A) In neutral conditions, the enzyme preferentially synthesizes cADPR by promoting a cyclization reaction. (B) In acidic conditions, the cyclase mostly produces NAADP the result of a base-exchange reaction (Bezin, Charpentier, Fossier, & Cancela, 2006).
**Figure 1-4:** Enzymatic pathway and cADPR production. cADPR is synthesized by ADP-ribosyl cyclase by cyclizing NAD while nicotinamide is released spontaneously. ADPR is produced from cADPR via cADPR hydrolase and/or from NAD through NAD glycohydrolase activity, respectively (Bai, Lee, & Laher, 2005).
Figure 1-5: Subunit structure and regulation of \( \text{Ca}_\alpha \text{1} \) channels. (A) The subunit composition and structure of \( \text{Ca}^{2+} \) channels purified from skeletal muscle are illustrated. The model is updated from the original description of the subunit structure of skeletal muscle \( \text{Ca}^{2+} \) channels (Takahashi et al 1987). P, sites of phosphorylation by cAMP-dependent protein kinase that have been demonstrated in intact cells (Catterall, 2000).
Figure 1-6: Domains of STIM proteins. *Drosophila* and human STIM proteins are situated in the ER membrane. Modules of STIM1, STIM2 and Stim include: the signal peptide, the predicted EF-hand and SAM domains, the transmembrane region and two regions predicted to form coiled-coil structures comprising the ERM domain. Proline-rich domains (P) and the lysine-rich C-terminal regions are unique to the mammalian STIM family members. *Drosophila* Stim contains an N-terminal sequence in the ER that is not present in either STIM1 or STIM2. The N-linked glycosylation sites at the SAM domain, experimentally verified for STIM1, are also indicated. Background colours represent basal Ca$^{2+}$ concentrations of ~50 nM in the cytosol and > 400 µM in the ER lumen. Ca$^{2+}$ ions are shown as red dots, including Ca$^{2+}$ bound to the EF-hand domain (Cahalan, 2009).
Figure 2-1: Carcinoid cell lines demonstrate a lower mitochondrial density compared to breast cancer cell lines. Wide-field fluorescence images of A, BON cells, B, CNDT2.5 cells, C, MCF-7 cells and D, MDA-MB231 cells transfected with Ds-Red mito (3) and loaded with Calcein (2). E, Quantification of mitochondrial density in all four cell lines shown as ratio values (arbitrary units) of red (Ds-Red mito) to green (Calcein) signal (n=5). Data represent one experiment.
Figure 2-2: Confocal images of A, BON cells, B, CNDT2.5 cells, C, MCF-7 cells and D, MDA-MB231 cells transfected with Ds-Red mito (3) and loaded with Calcein (2).
Figure 2-3: Mitochondria in carcinoid cell lines exhibit a lower membrane potential ($\psi_m$) in comparison to breast cancer cell lines. A, A confocal image of a CNDT2.5 cell loaded with JC-1. B, Quantification of $\psi_m$ among BON (n=9), CNDT2.5 (n=7), MCF-7 (n=9), and MDA-MB231 (n=8) cell lines loaded with JC-1. Data are represented as ratio values (arbitrary units) of red to green fluorescence signals ± SE. These data represent an accumulation of three separate experiments. C, Quantification of $\psi_m$ in BON cells loaded with JC-1 in the presence (white bar) and absence (black bar) of FCCP. These data represent an accumulation of two separate experiments.
Figure 2-4: The role of mitochondria in store-operated Ca\(^{2+}\) entry (SOCE). Wide-field fluorescence Ca\(^{2+}\) imaging of A, BON cells, B, CNDT2.5 cells, C, MCF-7 cells and D, MDA-MB231 cell lines loaded with fura-2 in the presence (red trace) and absence (black trace) of FCCP. Note the increased rate of rise of the Ca\(^{2+}\) transients in treated cells (A, B). E, Quantification of the peak amplitude of Ca\(^{2+}\) signals upon SOCE under control (black bars) and FCCP treated (white bars) conditions in carcinoid and breast cancer cells. F, Quantification of the rate of Ca\(^{2+}\) influx through SOCCs in the presence (white bars) and absence (black bars) of FCCP in carcinoid and breast cancer cell lines. Data are represented as mean half time to peak amplitude in seconds ± SE. Note the significant increase of rate in BON and CNDT cells upon FCCP treatment. These data represent an accumulation of three separate experiments.
E.

![Graph E](Image)

F.

![Graph F](Image)
Figure 2-5: TIRF (3,4) and wide-field images (1,2) of A, BON and B, MDA-MB231 cells transfected with Ds-Red mito and loaded with Calcein.

A.

B.
Figure 2-6: Carcinoids appear to be more resistant to Vit K₃/C treatment. Quantification of carcinoid and breast cancer cell viability upon Vit K₃/C treatment. Data are represented as the percent of control ± S.E. These data represent an accumulation of three separate experiments.
**Figure 2-7:** A, wide-field fluorescence image of CNDT2.5 cells loaded with CM-H$_2$DCFDA. B, Quantification of hydrogen peroxide (H$_2$O$_2$) production in carcinoid and breast cancer cell lines in the presence (white bar) and the absence (black bar) of Vit K$_3$/C mixture. Data are represented as mean values of CM-H$_2$DCFDA fluorescence intensity ± S.E. Note the significant increase in H$_2$O$_2$ level in all four cell lines. These data represent an accumulation of three separate experiments.
**Figure 2-8:** A, wide-field fluorescence image of MCF-7 cell line loaded with MitoSOX. B, Quantification of superoxide production in carcinoid and breast cancer cell lines in the presence (white bar) and the absence (black bar) of Vit K₃/C mixture. Data are represented as mean values of MitoSOX fluorescence intensity ± S.E. Note the significant increase in superoxide level in breast cancer cell lines. These data represent an accumulation of three separate experiments.
Figure 2-9: Quantification of $\psi_m$ of carcinoid and breast cancer cell lines in the presence (white bar) and absence (black bar) of Vit K$_3$/C mixture. Data are represented as ratio of red to green fluorescence signal ± S.E. These data represent an accumulation of three separate experiments.
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>cADPR</td>
<td>Cyclic ADP Ribose</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAMK-II</td>
<td>Calcium/calmodulin kinase II</td>
</tr>
<tr>
<td>CM-H₂DCFDA</td>
<td>5-(and-6)-chloromethyl-2′7′dichlorodihydrofluorescein diacetate acetyl ester</td>
</tr>
<tr>
<td>CoQ</td>
<td>Coenzyme Q</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclopiazonic acid</td>
</tr>
<tr>
<td>CRACM</td>
<td>Ca(2+) release-activated Ca(2+) channel modulator</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's Medium</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCCP</td>
<td>carbonylcyanide p-trifluoromethoxyphenylhydrazone</td>
</tr>
<tr>
<td>FKBP12</td>
<td>FK506-binding protein 12</td>
</tr>
<tr>
<td>FKBP12.6</td>
<td>FK506 binding protein 12.6</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-peptide f-Met-Leu-Phe</td>
</tr>
<tr>
<td>GI</td>
<td>GastroIntestinal</td>
</tr>
<tr>
<td>Glut</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIF1</td>
<td>Hypoxia-inducible factor 1</td>
</tr>
<tr>
<td>HVA</td>
<td>High-Voltage-Activated</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner Mitochondrial Membrane</td>
</tr>
<tr>
<td>InsP₃R</td>
<td>Inositol (1,4,5)-triphosphate receptor</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>LVA</td>
<td>Low-Voltage-Activated</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide(reduced form)</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane Ca2+ ATPase</td>
</tr>
<tr>
<td>PTP</td>
<td>Permeability transition pore</td>
</tr>
<tr>
<td>RaM</td>
<td>rapid mode</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RuR</td>
<td>Ruthenium Red</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptors</td>
</tr>
<tr>
<td>SAM</td>
<td>Sterile alpha motif</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/Endoplasmic Reticulum Ca2+-ATPase</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>SOC</td>
<td>Store-Operated Channel</td>
</tr>
<tr>
<td>SOCC</td>
<td>Store-operated calcium channels</td>
</tr>
<tr>
<td>SOCE</td>
<td>Store-operated calcium entry</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutases</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic Reticulum</td>
</tr>
<tr>
<td>STIM</td>
<td>stromal interaction molecule</td>
</tr>
<tr>
<td>TIRF</td>
<td>total internal reflection fluorescence</td>
</tr>
<tr>
<td>TK</td>
<td>tyrosine kinase</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>TPC</td>
<td>two-pore channels</td>
</tr>
<tr>
<td>TRPM2</td>
<td>transient receptor potential melastatin 2</td>
</tr>
<tr>
<td>UCP</td>
<td>uncoupling protein</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channels</td>
</tr>
<tr>
<td>VOCC</td>
<td>Voltage-Operated Calcium Channel</td>
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Chapter 1: Introduction (Literature Review)

In the current study, we compared mitochondrial function, \( \text{Ca}^{2+} \) dynamics and the effectiveness of mito-toxic intervention in human carcinoid and breast cancer cell lines. Our rationale was to compare and contrast the properties and role of mitochondria between indolent cancer cells (carcinoid) and highly aggressive, metabolically active (breast cancer) cell lines, and determine whether carcinoid cell mitochondria exhibited properties that defined them as susceptible targets for mito-toxic therapies. In this effort, the role of mitochondria in modulating \( \text{Ca}^{2+} \) entry via VOCCs and non-VOCCs was investigated using \( \text{Ca}^{2+} \) imaging techniques. Although message transcripts for VOCCs were identified in all carcinoid cell lines by RT-PCR methods, only one cell line expressed functional VOCCs. In contrast, breast cancer cells did not express VOCCs. Thus, we focused on a specific type of non-VOCC entry called store-operated \( \text{Ca}^{2+} \) entry or SOCE observed in all our cell lines.

We had previously shown that a selective SOCE blocker antagonized mitochondrial calcium import and thus local calcium clearance inhibited both SOCE and cell proliferation (Mignen et al., 2005). We now demonstrate that in carcinoid cell lines, the rates and the amplitudes of SOCE were altered by mitochondrial inhibition. The enhanced rate of rise of \( \text{Ca}^{2+} \) signals following SOCE activation suggested a close coupling between mitochondria and store-operated calcium channels in carcinoid cell. These findings are reminiscent of the compartmentalization of and cross talk between
mitochondria and store-operated channels (SOCs) others have observed in lymphocytes. In contrast, the rate of $\text{Ca}^{2+}$ entry was not significantly affected in breast cancer lines.

In addition, imaging techniques were applied to measure mitochondrial mass and energy state. Using mitochondrial targeted red fluorescent protein (RFP) in combination with Calcein (an indicator dye for cytoplasm), we measured mitochondrial density and found that there was reduced density and fewer mitochondria per cell in carcinoid cell lines in comparison to breast cancer lines. We also used JC-1, a semi quantitative dye (a reporter of mitochondrial membrane potential ($\psi_m$) to assess mitochondrial energy state. We observed that mitochondria of carcinoid cell lines are less polarized compared to the breast cancer cell lines. Moreover, in both carcinoid and breast cancer cells FCCP (carbonylcyanide-p-trifluoromethoxyphenylhydrazone), a potent uncoupler of oxidative phosphorylation collapsed mitochondrial membrane potential.

The experiments outlined above indicated that mitochondria in carcinoid cell lines are fewer in number and less energized. This could indicate that mitochondria in these cell lines may be more compromised in comparison to breast cancer cell lines and may be more susceptible to mito-toxic drug treatment as anti-cancer strategy.

To test this possibility we adopted an oxidative strategy that exploited vitamin K3 (also known as menadione) and vitamin C mixture (Vit K3/C) as an anti-cancer regimen in tumor cells. Several studies have shown that Vit K3/C generates a redox-cycling which can result in over-production of ROS. Mitochondria are both generators of ROS and targets of oxidative damage by ROS. Thus, Vit K3/C treatment was predicted to induce mitochondrial dysfunction, subsequent $\text{Ca}^{2+}$ homeostasis dysregulation and cell death.
Because many cancer cell lines have been shown to over express vitamin C transporters and concentrate vitamin C intracellularly, this strategy should provide oxidant challenge to cancer cells without affecting non-cancer cells.

We initially predicted carcinoids would be more susceptible to toxic effect of ROS given the lower energy state and diminished number of their mitochondria. However using cell growth assays, we found that breast cancer cells appeared to be more sensitive to the vitamin K3/C toxicity than carcinoids. To investigate this observation, ROS production in tumor cells was assessed using fluorescent dyes to measure H₂O₂ and superoxide production. Our results indicated that H₂O₂ production, thought to be the pathway that mediates cell death in Vit K3/C treated cells, was increased in both carcinoid and breast cancer cells, and thus did not correlate with cell toxicity. However, there were increased amounts of mitochondrial superoxide in breast cancer cell lines compared to carcinoid cell lines. Moreover, mitochondrial superoxide correlated with diminishment of cell number suggesting that superoxide rather than H₂O₂, mediated cell toxicity. Consistent with this idea, mitochondrial membrane potential was reduced by Vit K3/C for breast cancer cell lines and less so for carcinoid cell lines.

These data can explain why the treatment was more effective at reducing breast cancer cell number and raise important questions as to why carcinoids, that have fewer and less energized mitochondria than breast cancer cells, are more resistant towards treatment. These possibilities are discussed.
1.1 Calcium

Calcium, the most abundant mineral in the body plays a critical role in both extracellular and intracellular spaces. In fact the role of calcium in excitation-contraction coupling was discovered as early as the 1880s when Sydney Ringer observed that frog hearts would beat longer in vitro if the saline solutions were made from Ca$^{2+}$ containing “hard” water rather than from distilled water (Calcium, James W. Putney Jr., 1999). In addition, calcium serves an essential role in nerve impulse transmission, blood coagulation, hormone secretion, and intercellular adhesion. Calcium also is an important intracellular messenger for processes such as exocytosis, chemotaxis, enzymatic activity, gene transcription, cell proliferation and cell death (Berridge, Bootman, & Roderick, 2003).

A calcium signal is produced when a series of molecular and biophysical events link an external stimulus to the expression of some appropriate intracellular response through a sudden increase in cytosolic calcium. Calcium ions that give rise to calcium signals can originate from the following two sources: Intracellular stores (usually the endoplasmic reticulum (ER) and external media (Calcium, James W Putney Jr., 1999).

1.2 Calcium release from internal stores

To allow rapid and localized intracellular release of calcium, a steep gradient needs to be maintained across the sarcoplasmic/endoplasmic reticulum (SR/ER) membrane; this gradient is sustained by the SR/ER Ca$^{2+}$ ATPase pump (SERCA). Upon stimulation calcium is released via channels/receptors on the SR/ER membrane (calcium
release channels) which fall into two categories: the inositol 1, 4, 5-triphosphate receptors (IP3Rs) and the ryanodine receptors (RyRs). The difference between these two channel families and plasma membrane ion channels lies in their size and non-selective high conductance cation transport properties which allows the rapid release of SR/ER Ca\textsuperscript{2+} into the cytoplasm (Zalk, et al., 2007).

1.2.1 IP3Rs

The IP3 receptor which was first identified in the ER also resides in the Golgi complex, secretory granules, the plasma membrane, and the nucleoplasmic reticulum. All IP3R-containing membranes surround organelles with releasable calcium stores except for the plasma membrane where it can function as a scaffold for IP3R binding proteins. There are three types of IP3R. All these types of the IP3R are comprised of a tetramer where each unit contains a cytoplasmic NH2 terminus comprising 85% of the protein mass, a hydrophobic region predicted to contain six membrane-spanning helices that contribute to the ion-conducting pore of the IP3 channel, and a relatively short cytoplasmic COOH terminus. The IP3-binding region is localized in the N-terminus domain (Foskett, White, Cheung, & Mak, 2007)(Fig 1-1). Upon the binding of 1, 4, 5-triphosphate (IP3), the IP3R channel is gated open allowing calcium to diffuse down its electrochemical gradient from the ER to the cytoplasm. The secondary messenger IP3 is generated through the hydrolysis of the membrane phospholipid phosphoinositol-4, 5-biphosphate (PIP2) by the enzyme phospholipase C, or γ following G protein coupled receptor (GPCR) or receptor Tyrosine Kinase (TK) activation, respectively.
One way in which IP3R can be regulated is through phosphorylation. Multiple kinases including cyclic-AMP-dependent protein kinase (PKA) phosphorylate IP3R and regulate channel activity. IP3R can also be phosphorylated by protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CAMK-II) at different sites. PKC phosphorylation of IP3R leads to increased sensitivity to IP3-mediated Ca\(^{2+}\) release.

Another way in which IP3R is regulated is by Ca\(^{2+}\)-dependent modulation. IP3R regulation by calcium has been widely studied and is a biphasic process. A maximal effect of IP3R stimulation is achieved at about 100-300 nM calcium with inhibiting effects at low micromolar concentrations. The exact mechanism of calcium regulation needs further investigation, however, it is widely accepted that the calcium released initially by IP3R acts in a feedback manner to promote further calcium release. Conversely, high calcium concentrations inhibit channel function resulting in the classical oscillatory pattern of calcium release.

These observations underscore the complex interaction between Ca\(^{2+}\) and IP3. For example, IP3 regulates IP3R channel activity by simply modulating IP3R sensitivity to Ca\(^{2+}\) inhibition. Although spontaneous IP3R activity has been reported, both appropriate levels of Ca\(^{2+}\) and IP3 must be present on the cytoplasmic side of the channel to result in detectable channel activity. IP3 and Ca\(^{2+}\) however, regulate IP3R in distinct manners. Lower levels of IP3 (subsaturating levels) in the presence of appropriate Ca\(^{2+}\) concentrations makes the IP3R more sensitive to inhibition.

Also, IP3R function is influenced by ATP independently of phosphorylation or energy dependent processes. In vitro, ATP has been shown to enhance IP3-induced
calcium flux at about 100 uM ATP. High concentrations of ATP, however, inhibit IP3-induced release of calcium which is probably due to direct competition for the ligand binding pocket. IP3R is also regulated by the adenine nucleotide NADH. At physiologic concentrations, NADH promotes IP3R-mediated calcium flux (Patterson, Boehning, & Snyder, 2004).

1.2.2 RyRs

The second category of calcium release channels are the Ryanodine receptors (RyRs). There are three known isoforms of RyRs (RyR1, RyR2 and RyR3) encoded by three distinct genes. RyR1 is predominantly expressed in skeletal muscles whereas RyR2 is expressed in cardiac muscle and the brain. RyR3 is found in smooth muscle, the diaphragm, as well as the brain. In neurons all three isoforms are present. Subcellularly RyRs are present in specialized portions of the endoplasmic/sarcoplasmic reticulum membranes.

Ryanodine receptors are homotetrameric complexes with the four subunits forming a square around the central core and a total mass of approximately 2200 kDa. The N-terminus of the RYRs consists of a large cytoplasmic domain that modulates gating of the channel pore which is located in the C terminus (Fig 1-2).

The intracellular Ca\(^{2+}\) release in skeletal and cardiac muscles is directly controlled by RyR channels to achieve muscle contraction. RyRs function as a macromolecular signaling complex which includes kinases and phosphatases associated with the cytoplasmic channel region. The channel activity is triggered upon extracellular signals
via second messengers to ultimately regulate ER/SR Ca\textsuperscript{2+} release. Ca\textsuperscript{2+} binding can activate all RyR isoforms, although RyR1 can be activated mechanically through conformational changes.

In addition to conformational changes evoked by voltage-operated calcium channels (VOCC) gating, the function of RyRs can be modulated by a variety of agents including Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, ATP, cADPR (Cyclic ADP-Ribose), Calmodulin, FKBP12, post-translational modifications, and pharmacologic substances such as ryanodine and caffeine.

For example, Ca\textsuperscript{2+} regulation of RyRs can vary by RyR isoforms. RyR1 activity depends on Ca\textsuperscript{2+} concentration changes of which can either lead to RyR1 activation or inhibition suggesting that RyR1 has at least two Ca\textsuperscript{2+} binding sites. RyR1 and RyR2 are both activated at 0.1-10 Mm Ca\textsuperscript{2+} though higher Mg\textsuperscript{2+} levels seem to affect RyR1 inhibition more, i.e., lower Ca\textsuperscript{2+} concentrations activate the channel while higher concentrations inhibit channel activity. Decrease of SR Ca\textsuperscript{2+} on the luminal side inactivates RyR2.

In addition, cytosolic Mg\textsuperscript{2+} is a potent inhibitor of RyR channels. Modulation of RyRs by Mg\textsuperscript{2+} decreases Ca\textsuperscript{2+} sensitivity. Moreover, under physiological conditions, ATP can contribute to RyR activity. In the presence of Ca\textsuperscript{2+} ATP has been shown to increase single-channels RyR2 activity. Furthermore, the RyR1 is the major CaM-binding protein of the SR membrane. Ca\textsuperscript{2+} at uM concentrations leads to CaM inhibition of both RyR1 and RyR2. Lower concentrations of Ca\textsuperscript{2+} however lead to CaM activation of RyR1 while inhibiting RyR2 (Zalk, et al., 2007).
1.3 Other putative Ca\textsuperscript{2+} mobilizing receptors

Although IP3 and Ca are the universally accepted second messengers, other crucial signaling molecules have also been implicated in the Ca\textsuperscript{2+} signaling process.

1.3.1 NAADP

Nicotinic acid adenine dinucleotide phosphate (NAADP) was shown to be a potent Ca\textsuperscript{2+} mobilizing messenger initially in sea urchin eggs. The role of NAADP in Ca\textsuperscript{2+} signaling now has been demonstrated in a variety of mammalian cell lines such as neurons, cardiac myocytes, smooth muscle, exocrine cells, and platelets (Galione, et al., 2009). The NAADP action in mammalian cells was first demonstrated in pancreatic acinar cells in which NAADP initiated cholecystokinin-induced Ca\textsuperscript{2+} signals (Bezin, et al., 2006).

Structurally, NAADP is very similar to nicotinamide adenine dinucleotide phosphate (NADP), the well-known co-enzyme, with the only difference being the conversion of the nicotinamide group to a carboxyl group. This slight change of structure has made NAADP a highly potent intracellular messenger (Fig 1-3).

NAADP is synthesized in response to poorly defined cellular stimuli and has the ability to release Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores. There is also some evidence that NAADP may be involved in Ca\textsuperscript{2+} influx (Higashida, et al., 2007; Moccia, Lim, Nusco, Ercolano, & Santella, 2003). Two enzymes, Aplysia ADP-ribosyl cyclase and its mammalian homolog CD 38, so far have been shown to be responsible for the NAADP synthesis (Lee, 2005).
The search for target organelle and the target receptor for NAADP is a subject of intense study. It has been suggested that lysosome-related acidic organelles are the target for NAADP-induced Ca\(^{2+}\) mobilization in various cell types. Lysosomal localized cation channels have been tested for NAADP binding or an NAADP-induced Ca\(^{2+}\) response. A very recent work implicated that two-pore channels (TPCs or \(TPCNs\) for gene names) may function as NAADP receptors. TPCs belong to the super family of voltage-gated cation channels. Structural analysis of a full length protein revealed 12 putative transmembrane (TM) segments that are segregated into two homologous domains of six TM segments each with an intervening hydrophobic loop between the fifth and the sixth segments (Galione, et al., 2009).

Although studies suggest TPCs as NAADP-regulated Ca\(^{2+}\) release channels, other candidate channels may also have a role in NAADP-induced Ca\(^{2+}\) mobilization.

Additionally, it has been reported that the NAADP receptor possesses the property of self-desensitization by NAADP which can be achieved at various NAADP concentrations depending on cell type (Bezin, et al., 2006).

1.3.2 cADPR

In 1987, Lee and coworkers first identified cyclic adenosine diphosphate ribose (cADPR) as a calcium mobilizing messenger in sea urchin eggs (Bai, et al., 2005). Variety of cell types have been reported to respond to cADPR including smooth, skeletal and cardiac muscle cells, neuronal and neuronal-related cells, hematopoietic cells, acinar cells, and oocytes (Guse, 2005).
cADPR has a complete circular structure and is formed by head-to-tail linkage of a long linear molecule of nicotinamide adenine dinucleotide (NAD) while the terminal nicotinamide group in NAD is released in the process (Fig 1-4). It is interesting to note that cADPR is a very stable molecule. At room temperature, it takes days for cADPR to spontaneously hydrolyze to ADPR (Bai, et al., 2005). ADP-ribosyl cyclases, the same class of enzymes that produce NAADP, can form cADPR upon stimulation by extracellular stimuli or metabolic activity. *Aplysia* ADP-ribosyl cyclase and CD38 (the mammalian homologue) are the two known cADPR cyclizing enzymes.

It has been shown that cADPR directly binds to types II and III ryanodine receptors to initiate its Ca\(^{2+}\) releasing function. Also there is growing evidence that cADPR may function through its specific receptor proteins such as FK506 binding protein 12.6 (FKBP12.6) which bind to type II ryanodine receptors. Dissociation of the binding protein from the ryanodine receptor is thought to activate or delay inactivation of the receptor.

In addition to calcium release via RyR, cADPR has been demonstrated to activate calcium entry. Evidence for cADPR role in calcium entry was obtained in human T-lymphocytes and neutrophils. In T-lymphocytes, membrane-permeant cADPR antagonist 7-deaza-8-Br-cADPR as well as antisense RNA-mediated knock down of type 3 RyR blocked sustained entry of Ca\(^{2+}\). Furthermore, in neutrophils from wild type mice, the chemotactic peptide fMLP induced biphasic calcium signaling – calcium release followed by calcium entry (Fliegert, Gasser, & Guse, 2007).
Two possible underlying mechanisms for cADPR-induced Ca\textsuperscript{2+} entry exist:
capacitative Ca\textsuperscript{2+} entry mechanism which is generally assumed to play a role in nonexcitable cells. Evidence obtained from RyR knock-down Jurkat T cells suggests that cADPR-mediated store depletion may activate store-operated Ca\textsuperscript{2+} entry. On the other hand, it was recently shown that cADPR may be involved in Ca\textsuperscript{2+} entry through activation of TRPM2 channel. The plasma membrane ion channel transient receptor potential – melastatin-like (TRPM2) is a Ca\textsuperscript{2+} – and Na\textsuperscript{+} –permeable cation channel that is mainly expressed in the brain and in the cells of the immune system (Guse, 2005).

### 1.4 Calcium influx

Two primary ways in which Ca\textsuperscript{2+} can enter the cell are via voltage-operated Ca\textsuperscript{2+} channels (VOCC) and/or non-voltage-operated Ca\textsuperscript{2+}-permeable channels.

#### 1.4.1 Voltage-operated calcium channels

Voltage-operated Ca\textsuperscript{2+} channels (VOCC) are activated upon membrane depolarization. A variety of Ca\textsuperscript{2+} currents with distinct characteristics have been identified suggesting the existence of two major classes of VOCC: high-voltage-activated (HVA) and low-voltage-activated (LVA) Ca\textsuperscript{2+} channels. HVA Ca\textsuperscript{2+} channels generally possess large conductances, are activated by strong depolarizations and exhibit variable inactivation kinetics. In contrast, LVA channels typically maintain a small conductance (8-12 pS), are activated by small changes from the resting membrane potential and inactivate rapidly.
HVA consists of L-type, N-type, PQ type, and R-type channels. These are described below.

1.4.1A L-type

L-type currents are the major Ca\(^{2+}\) currents in cardiac, smooth, and skeletal muscles and distinguished by high voltage of activation, large single-channel conductance, slow voltage-dependent inactivation, marked regulation by cAMP-dependent protein phosphorylation pathways and specific inhibition by Ca\(^{2+}\) antagonist drugs such as dihydropyridines. They were also recorded in endocrine cells and neurons. Typically, L-type currents are activated at membrane potentials above -30mV and generally exhibit slower inactivation properties. They play an important role in muscle contraction, gene transcription, endocrine secretion, and integration of synaptic inputs, and are regulated primarily by direct interaction of G proteins and SNARE proteins and secondarily by protein phosphorylation. These Ca\(^{2+}\) currents have been designated as L-type as they are long lasting when Ba\(^{2+}\) is the current carrier.

1.4.1B N-Type

N-type currents are distinguished by their intermediate voltage dependence and rate of inactivation. They are activated at more negative potentials and display faster kinetics compare to L-type currents but are activated at more positive potentials and are slower than T-type currents. N-type currents can be blocked by the cone snail peptide \(\omega\)-conotoxin GVIA which has been the primary method to distinguish them from other
currents because their kinetics and voltage dependence vary considerably in different neurons.

1.4.1C  P-, Q-, and R-type

Pharmacological analysis revealed three additional Ca\(^{2+}\) currents. P-type currents were first recorded in Purkinje neurons and are highly sensitive to the spider toxin \(\omega\)-agatoxin IVA. First, recorded in cerebellar granule neurons, Q-type Ca\(^{2+}\) currents are also blocked by \(\omega\)-agatoxin IVA but with lower affinity. R-type currents are resistant to the subtype-specific organic and peptide Ca\(^{2+}\) channel blockers and may include multiple channel subtypes. They are most prominent in neurons.

LVA currents are comprised of the T-type family of channel proteins

1.4.1D  T-type

Voltage-clamp studies of Ca\(^{2+}\) currents in starfish eggs and recordings of Ca\(^{2+}\) action potentials in cerebellar Purkinje neurons revealed Ca\(^{2+}\) currents with different properties form L-type currents. They are called T-type for their transient kinetics or low voltage-activated for their negative voltage dependence. T-type currents have small-channel conductance, are inactivate rapidly, deactivate slowly, and are insensitive to Ca\(^{2+}\) antagonist drugs
1.4.2 Structural properties of VOCC proteins

Voltage-dependent channel is a complex of a pore-forming α1 subunit of ~190-250 kDa; a transmembrane, disulfide-linked α2β dimer of 170 kDa, an intracellular β subunit of 55 kDa; and in some cases a 33 kDa transmembrane γ subunit (Fig 1-5). The α1 subunit is a protein of about 2000 aminoacid residues which are organized in four repeated domains. Each domain contains six transmembrane segments (S1 to S6) and a membrane-associated loop between transmembrane segments S5 and S6. The β subunit contains α helices but has no transmembrane segments. The γ subunit which is a glycoprotein is reported to be composed of four transmembrane segments. Studies have shown that α2 subunit, however, is an extracellular protein that is anchored to the membrane through a disulfide linkage to the δ subunit. Studies performed on the cloned α2 subunit have indicated the presence of many glycosylation sites and hydrophobic sequences. The mature forms of both the α2 and δ subunits are formed by post-transitional proteolytic processing and disulfide linkages. Interestingly, the δ subunit is a product of the 3’ end of the coding sequence of same gene that encodes the α2 subunit. To date, ten different types of α1 subunits, four types of α2 δ complexes, four types of β subunits, and two different types of the γ subunits have been identified.

Functional analysis of Ca\(^{2+}\) channel expressed subunits was first carried out with skeletal muscle channels where the expression of the α1 subunit sufficed to form functional Ca\(^{2+}\) channels. However, the α1 subunit was expressed in low levels and the Ca\(^{2+}\) current was associated with abnormal kinetics and voltage dependence. Interestingly, co-expressing the other subunits such as the α2δ complex and specially the
β subunit results in increased expression levels of the α1 subunit thereby, rendering channels with more normal gating properties. Similar observations have been observed with the Ca\(^{2+}\) channels in cardiac and neuronal Ca\(^{2+}\) channels co-expressing the β subunits suggesting a general increase in the expression levels of the α1 subunit which in turn results in increased functionality of the Ca\(^{2+}\) channels.

The different types of Ca\(^{2+}\) currents are primarily defined by different α1 subunits. These subunits were originally designated classes A through I, and more recently a nomenclature dividing the Ca\(^{2+}\) channels into three structurally and functionally related families (Cav1, Cav2, and Cav3) has been proposed. L-type Ca\(^{2+}\) currents are mediated by Cav1 family of α1 subunits, which have about 75% amino acid sequence identity with the α1 subunit of skeletal muscle L-type Ca\(^{2+}\) channels. The Cav2 channels form a distinct family with less than 40% amino acid sequence identity with Cav1 α1 subunits but greater than 70% amino acid sequence identity among themselves. The Cav2 family conducts P/Q, N-, and R-type Ca\(^{2+}\) currents. T-type Ca\(^{2+}\) currents are mediated by the Cav3 channels (Catterall, 2000; Jones, 2003).

1.4.3 **Store-operated Ca\(^{2+}\) entry (SOCE)**

Store-operated Ca\(^{2+}\) channel is one of the most studied of the non-voltage activated channels. The concept of capacitative or store-operated Ca\(^{2+}\) entry (SOCE) was first introduced by James Putney in 1986. The depletion of intracellular Ca\(^{2+}\) stores can trigger Ca\(^{2+}\) influx through plasma membrane-localized Ca\(^{2+}\) channels known as store-operated Ca\(^{2+}\) channels (SOCC)(Cahalan, 2009). Ca\(^{2+}\) entry through SOCCs is essential for the replenishment of the Ca\(^{2+}\) stores and is also involved in cellular Ca\(^{2+}\) homestasis.
In many cell types, including hematopoietic cells, SOCs carry a highly Ca$^{2+}$-selective, non-voltage-gated Ca$^{2+}$ current which was first described by Hoth and Penner in RBL mast cells and termed the Ca$^{2+}$ release-activated Ca$^{2+}$ current, or I$_{crac}$ (Cahalan, 2009).

Molecular mechanism of SOCE remained undefined until recently. STIM proteins, as the molecular link from ER Ca$^{2+}$ store depletion to SOCE and CRAC channel activation, and Orai (CRACM) proteins which comprise the CRAC channel pore-forming subunit were identified using RNAi screening. Mammals have two STIM proteins, STIM 1 and 2, and three Orai proteins, Orai 1, Orai 2, and Orai 3, which are ubiquitously expressed in different cell types. STIM 1 and its structurally related homologue STIM 2 are type 1 transmembrane proteins. STIM 1 primarily resides in the ER but can be found to a limited extent in the PM however STIM 2 can only be found in the ER. STIM 1 contains multiple discrete regions including an EF-hand and a sterile α-motif domain (SAM) in the N-terminus directed towards the lumen of the ER. In addition to a transmembrane region, STIM 1 also contains a coiled-coil domain, a serine-proline region, and a lysine-rich region in the C-terminus facing the cytoplasmic side (Fig 1-6).

The presence of an EF-hand motif near the N-terminus of STIM1 suggested a role in sensing ER Ca$^{2+}$ (Cahalan, 2009). When the ER Ca$^{2+}$ stores are filled, STIM is a dimer stabilized by C-terminal coiled-coil interactions. Upon the store depletion, STIM1 rapidly clusters and relocalized to PM-adjacent ER regions. Formation of these clusters is initiated by decreased binding of Ca$^{2+}$ to the EF hand causing the oligomerization of
STIM1 followed by translocation of the multimers to membrane-adjacent ER areas where they can activate Ca\(^{2+}\) influx.

Mutational analysis has shown that Orai1 could be the channel component of SOCE. Cross-linking studies indicate that Orai proteins form dimers in the resting state. It is hypothesized that two Orai dimmers cluster with cytoplasmic domain of STIM1 following STIM1 oligomerization to form a functional channel once ER Ca\(^{2+}\) drops. (Varnai, Hunyady, & Balla, 2009).

1.5 Mitochondria and calcium dynamics

1.5.1 Mitochondria

Mitochondria are specialized double membrane-bound organelles that are essential for a variety of cellular functions including growth, division, energy metabolism and apoptosis (Capiod, Shuba, Skryma, & Prevarskaya, 2007). Often called the “powerhouse of the cell”, mitochondria are the main sites for ATP production that is required for cell functioning and survival and is produced via the activity of electron transport chain (ETC) located in the inner membrane of mitochondria. Electrons are passed through the ETC from an electron donor (NADH or QH\(_2\)) to a terminal electron acceptor (O\(_2\)) via a series of redox reactions resulting in the generation of membrane potential (Ψ\(_m\)).

Ψ\(_m\) used for ATP synthesis is also the main driving force for Ca\(^{2+}\) accumulation across the inner mitochondrial membrane.
1.5.2 Mitochondria and Ca $^{2+}$ dynamics

Calcium concentration in the cytosol ([Ca$^{2+}]_c$) varies dynamically under the tight control of coordinated homeostatic mechanisms (Murgia, Giorgi, Pinton, & Rizzuto, 2009). It should be remembered that while the absolute resting values of the Ca$^{2+}$ in the cytosol and in the stores are determined by the balance of the activities of pumps and channels, the dynamic changes are strongly influenced by the presence of buffering systems. Organelles such as mitochondria, peroxisomes and secretory vesicles accumulate part of the Ca$^{2+}$ released from the stores, thereby acting as fixed buffers (Murgia, et al., 2009).

In particular, mitochondria have been shown to be critical for Ca$^{2+}$ buffering and local ATP production (which is crucial for ER/SR Ca$^{2+}$ accumulation and/or PMCA Ca$^{2+}$ extrusion) and thus cytosolic Ca$^{2+}$ dynamics via their ability to sense and shape calcium signals (Demaurex, Poburko, & Frieden, 2009). Mitochondria are suggested to have a low affinity and a high capacity for Ca$^{2+}$. As Ca$^{2+}$ rises above a certain point (>500 nM), mitochondria start to accumulate Ca$^{2+}$. At this point, Pi-dependent Ca$^{2+}$ buffering mechanism counterbalances the rise in [Ca$^{2+}]_m$.

1.5.3 Ca$^{2+}$ uptake from the extramitochondrial space

Ca$^{2+}$ has to cross two membranes to enter mitochondrial matrix; inner and outer mitochondrial membranes (IMM and OMM). Several studies have shown that voltage-dependent anion channel located on the outer mitochondrial membrane may play a role in Ca$^{2+}$ uptake. However, studies on VDAC knock down cells suggest that other Ca$^{2+}$
uptake pathways may exist through OMM. Mitochondrial sensitivity to Ca\(^{2+}\) remained unaltered in these cells compared to their wild type counter parts. Also, Bid a proapoptotic of Bcl2 family may be important for Ca\(^{2+}\) uptake (Szabadkai & Duchen, 2008).

\[ \text{Ca}^{2+} \text{ enters mitochondria through IMM via a uniporter. The molecular identity of this pathway remains obscure but properties are reasonably well characterized. Ca}^{2+} \text{ uptake is inhibited by ruthenium red (RuR), a cation channel inhibitor. Patch-clamp studies have also suggested the existence of the Ca}^{2+} \text{ uniporter. The uniporter is dependent on extramitochondrial Ca}^{2+} \text{ concentration. Montero et al. showed that an increase in } [\text{Ca}^{2+}]_c \text{ promoted RuR-sensitive (and thus uniporter-mediated) Ca}^{2+} \text{ release when mitochondria were depolarized.} \]

As mentioned earlier, the molecular identity of the uniporter is still unknown. However, there are a number of candidates. It has recently been suggested that uncoupling proteins UCP-2 and -3 may be essential for Ca\(^{2+}\) uptake either as the components of the channel itself or as part of a complex modulating the uptake (Szabadkai & Duchen, 2008).

Alternatively, another study indicated that heart mitochondria contain a functional RyR within the IMM which appears to be essential for Ca\(^{2+}\) uptake during Ca\(^{2+}\) oscillations. mRyR shares similar biochemical, pharmacological, and physiological characteristics with both the SR-RyR and the uniporter. For example typically, Ca\(^{2+}\) rises in the cytoplasm can activate RyR in the SR or ER to release Ca\(^{2+}\) from the intracellular stores. Conversely, in the case of a mitochondrial RyR, the inwardly directed
Ca\textsuperscript{2+} electrochemical gradient in mitochondria could result in Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} uptake (Beutner, Sharma, Giovannucci, Yule, & Sheu, 2001).

Another uptake mechanism with properties distinct from those of the uniporter has also been described. It is called rapid uptake mode (RaM) mechanism. This pathway can rapidly transfer Ca\textsuperscript{2+} into mitochondria during the rising phase of a Ca\textsuperscript{2+} pulse. The functional significance of RaM is remained to be established (Szabadkai & Duchen, 2008). RaM was reported in isolated liver mitochondria exposed to physiological Ca\textsuperscript{2+} pulses lasting 1-10s, hundreds of times faster than the Ca\textsuperscript{2+} uptake by the uniporter. Only at the beginning of the Ca\textsuperscript{2+} pulses, RaM was transiently activated. However, it was rapidly recovered between pulses, enabling mitochondria to respond to repetitive Ca\textsuperscript{2+} transient. The rapid mode was detectable already when [Ca\textsuperscript{2+}]\textsubscript{c} increased above 200 nM and thus did not require mitochondria to be in proximity to high Ca\textsuperscript{2+} microdomains (Gunter & Sheu, 2009).

1.5.4 Ca\textsuperscript{2+} extrusion into the surroundings

There are multiple pathways which restore mitochondrial Ca\textsuperscript{2+} levels. For example, the dominant route for Ca\textsuperscript{2+} efflux from mitochondria in most cells is through a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Its molecular identity is distinct from the plasma membrane exchanger and is still unknown (Graier, Frieden, & Malli, 2007). Located in the inner mitochondrial membrane, the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger mediates the efflux of Ca\textsuperscript{2+} from the mitochondria coupled to the influx of Na\textsuperscript{+}; a stoichiometry of 3 Na\textsuperscript{+}/Ca\textsuperscript{2+} was suggested (Murgia, et al., 2009). The operation of the exchanger is dependent on ∆Ψ\textsubscript{m} supported by
the observation that mitochondrial depolarization leads to inhibition of mitochondrial Ca\(^{2+}\) efflux (Szabadkai & Duchen, 2008).

Another exchange mechanism initially described in liver mitochondria is H\(^{+}/Ca^{2+}\) pathway (Graier, et al., 2007). A functional H\(^{+}/Ca^{2+}\) exchanger requires transmembrane potential in order to extrude Ca\(^{2+}\) from mitochondria against its gradient. It saturates at low calcium loads and its kinetics is extremely slow.

Located in the inner mitochondrial membrane, permeability transition pore (PTP), is considered as an additional important pathway for Ca\(^{2+}\) extrusion in mostly defined pathological conditions. PTP opening can be induced by intramitochondrial Ca\(^{2+}\) while ATP, ADP, Mg\(^{2+}\), and cyclosporine A inhibit it. It has been proposed that PTP can clear the mitochondrial matrix of damaged or unneeded molecules; permeability transition could also provide an important pathway for inducing apoptosis or for removing damaged mitochondria. Indeed, opening of a large non-selective pore that can allow molecules as large as 1.5 kDa in the inner mitochondrial membrane would allow maximal Ca\(^{2+}\) efflux, due to the collapse of the membrane potential and guarantee fast Ca\(^{2+}\) release even for very small [Ca\(^{2+}\)] gradients (Murgia, et al., 2009).

1.5.5 Mitochondria as a regulator of Ca\(^{2+}\) entry pathways

Mitochondria have been shown to modulate Ca\(^{2+}\) influx via VOCC and non-VOCC. Although, the role of mitochondria in VOCC-mediated Ca\(^{2+}\) entry has been studied, the focus of the current study is the role mitochondria play in SOCE. Others including our lab have shown that mitochondria can modulate the activity of store-
operated Ca\(^{2+}\) channels by buffering Ca\(^{2+}\) ions or by supplying ATP and other soluble factors (Demaurex, et al., 2009; Mignen, et al., 2005).

It was reported for the first time in 1997 that functional mitochondria are required to allow a sustained SOCE in T lymphocytes (Demaurex, et al., 2009). Earlier as it was indicated by several studies, mitochondria were required to sustain the CRAC currents but were not necessary for CRAC activation. Mitochondrial inhibition with FCCP or antimycin A in Jurkat T cells led to a transient increase in cytosolic Ca\(^{2+}\) following store depletion by subsequent inhibition of the sustained phase. However, the role of mitochondria as Ca\(^{2+}\) buffers in other cell types suggested reduced mitochondrial Ca\(^{2+}\) uptake altered both the activation and the sustained phase of SOCE in endothelial cells, Hela cells, and myo tubes.

It was proposed that mitochondria are located close to the Ca\(^{2+}\) entry channels in order to efficiently buffer Ca\(^{2+}\). (Quintana, et al., 2007) showed that some mitochondria translocate to the PM during Ca\(^{2+}\) entry and take up large amounts of Ca\(^{2+}\) in lymphocytes. Interestingly, it was shown in HeLa cells that relocation of mitochondria away from PM had no consistent effect on SOCE although functional mitochondria were still required for full SOCE activation. Indeed, it was proposed that mitochondria may release compounds that act positively on CRAC (Demaurex, et al., 2009). Recently it was shown in Jurkat T cells that ATP produced by mitochondria efficiently buffers Ca\(^{2+}\) and thus prevents the slow Ca\(^{2+}\)-dependent inactivation of the CRAC. ATP can diffuse and exert buffering actions at more distant sites from mitochondria (Quintana, et al., 2007).
The discovery of STIM1 and Orai1 as major molecular players in SOCE places mitochondria as regulators of Orai currents. In contrast, mitochondria were not reported to be involved in the regulation of fast Ca\(^{2+}\)-dependent inactivation of SOCE. (Bakowski & Parekh, 2007) showed recently that pyruvate (metabolized within the mitochondria) reduced the extent of the fast Ca\(^{2+}\)-dependent inactivation of SOCE.

The third regulation of SOCE by mitochondria is linked to the level of store depletion achieved during cell stimulation. Recently Parekh proposed that mitochondria initially promote store depletion and subsequently favor ER refilling, thus playing a role at each end of the SOCE process. The role of mitochondria in simply supplying ATP should not be underestimated. Ca\(^{2+}\) pumping by both SERCA and PMCA is ATP-dependent. Mitochondria can activate SOCE by supplying ATP to the PMCA thus shifting Ca\(^{2+}\) ions from the lumen of the ER to the extracellular solution. In contrast, by energizing SERCA, mitochondria favor store refilling and prevent SOCE activation. Whether mitochondria enhance or reduce the level of ER depletion depends on the spatial organization of the organelles and on the relative contribution of mitochondria. For example, in buffering Ca\(^{2+}\) near Ca\(^{2+}\) release or Ca\(^{2+}\) entry channels and in supplying ATP for SERCA and PMCA (Demaurex, et al., 2009).

1.6 Mitochondria and ROS

Reactive oxygen species (ROS) as by-products of aerobic metabolism are involved in a variety of cellular processes including proliferation, adaptation to hypoxia, apoptosis, carcinogenesis, and as intracellular messengers ROS can modulate signal transduction pathways (Batandier, Fontaine, Keriel, & Leverve, 2002). Under
physiological conditions, antioxidant capacity neutralizes ROS production. But when an excess of ROS is generated (ETC dysfunction, ischemia/reperfusion, apoptotic process) or if the antioxidant defense is overwhelmed (malnutrition, genetic disease, neurodegenerative disease), oxidative stress is created corresponding to the detrimental effect of ROS. Oxidative stress resulting from redox state dysregulation has been implicated in the pathogenesis of many diseases including cancer (Batandier, et al., 2002).

Relevant to the current study, mitochondria are acknowledged as both a major source and a target of ROS production and damage. Mitochondrial oxidative phosphorylation pathway is a permanent source of ROS in cells. About 1 to 3% of total mitochondrial oxygen consumed is incompletely reduced and leads to ROS production. Superoxide (O2•−) is generated by the transfer of one single free electron to molecular oxygen at the level of NADH CoQ reductase (Complex I) and CoQ cytochrome C reductase (Complex III). This superoxide anion is scavenged by intramitochondrial Superoxide Dismutase (MnSOD) leading to hydrogen peroxide (H2O2) and in the presence of reduced transition metals (such as iron) to the highly reactive hydroxyl radical (OH•). It is generally accepted that following its generation in the inner mitochondrial membrane, at the level of the respiratory chain, superoxide anions are vertically released into the mitochondrial matrix and subsequently dismutated in hydrogen peroxide by the high-level of matrix enzyme MnSOD. Contrary to the low diffusion capacity of the charged superoxide anion, hydrogen peroxide can easily cross the mitochondrial membrane towards cytoplasm. It should be added that a direct release of superoxide anion into the intermembrane space has been recently proposed.
Similarly to the mitochondrial super oxide ion pathway, in the cytosol the scavenging of $O_2^\cdot$ to $H_2O_2$ is permitted by an equivalent enzyme: CuZn SOD. $H_2O_2$ can be further broken down to $H_2O$ and $O_2$ by the enzyme catalase.

Thus, the deleterious effects of ROS can be counteracted by several mechanisms permitting an efficient antioxidant defense. However, if these defenses are compromised, mitochondria often represent the first target.

Alterations of mitochondrial membrane potential are the consequence of lipid peroxidation and thiol residue damage. Mitochondrial DNA is particularly sensitive to ROS because of the lack of histone protective system as is the case of nuclear DNA. These alterations result in mitochondrial dysfunction with decreased ATP production (ROS injury on ETC), $Ca^{2+}$ dysregulation, membrane permeabilization (ROS injury on lipid membrane), and mtDNA rearrangements (Batandier, et al., 2002).

### 1.6.1 Mitochondrial $Ca^{2+}$ overload and ROS production

Mitochondria actively contribute to the spatiotemporal profiles of intracellular $Ca^{2+}$ under both physiological and pathological conditions. $Ca^{2+}$ particularly at the high concentrations experienced in pathological conditions, appears to have several negative effects on mitochondrial function. For instance, The PTP is triggered by high $[Ca^{2+}]_m$.

Several studies have linked the PTP opening to cytochrome $c$ release and apoptosis. It has been shown that *cytochrome c* release and apoptosis is inhibited by PTP inhibitors (e.g., cyclosporin A), and VDAC (part of the PTP complex) has been shown to interact with proapoptotic Bcl family Proteins.
Importantly, PTP opening is accompanied by a burst of ROS which has been suggested to play a role in the autoamplification phase of the pore. It has been shown that PT pore triggering by ROS is potentiated by Ca\textsuperscript{2+}. This is an example of the two-hit hypothesis in which the combination of Ca\textsuperscript{2+} plus a pathological stimulus such as ROS can elicit mitochondrial dysfunction.

Moreover, recent data has suggested that cytochrome c can bind to IP3R rendering the channel insensitive to autoinhibition by high [Ca\textsuperscript{2+}]\textsubscript{c} and resulting in enhanced ER Ca\textsuperscript{2+} release. The close proximity between the ER and mitochondria facilitates this cross-talk.

By stimulating TCA cycle, Ca\textsuperscript{2+} would enhance ROS output by making the whole mitochondrion work faster and consume more O\textsubscript{2}. Indeed, mitochondrial ROS generation correlates well with metabolic rate, suggesting that a faster metabolism simply results in more respiratory chain electron leakage.

Another possibility is that Ca\textsuperscript{2+} can perturb mitochondrial antioxidant status. It has been observed that mitochondrial GSH is released very early in Ca\textsuperscript{2+} -induced PTP opening, suggesting that Ca\textsuperscript{2+} -exposed mitochondria may generate more ROS because of diminished GSH levels (Brookes, Yoon, Robotham, Anders, & Sheu, 2004)

1.7 Mitochondria and cancer: An oxidative strategy

The involvement of mitochondria in cancer cell metabolism, functions, and therapeutic potential is well documented. For example, a glycolytic phenotype is a major characteristic of cancer cells. This up-regulation of glycolysis is often associated with
hypoxia, overexpression of mitochondrial enzymes, and increased occurrence of mitochondrial defects (Verrax, et al., 2009). Moreover because the glucose transporter is a path for vitamin C entry, cancer cells commonly accumulate vitamin C and exhibit poor antioxidant status predisposing cancer cell to increased oxidative stress (Verrax, Taper, & Buc Calderon, 2008). Vitamin C or ascorbic acid is a potent water soluble antioxidant which possesses a wide variety of functions. For example, as a cofactor in a number of hydroxylation reactions, vitamin C can transfer electrons to the enzymes that provide reducing equivalents. Despite the existence of sodium-dependent transporters 1 and 2 (SVCT1 and SVCT2) as its transporters, most cells transport vitamin C as DHA (the oxidized form of ascorbate) via glucose transporters (Glut), including Glut1. Glut1 is ubiquitously expressed in cells and up-regulated likely through the activation of HIF1 expression program. Since DHA is rapidly reduced on the internal side of the plasma membrane, this prevents its efflux and allows the accumulation of ascorbate against a concentration gradient (Verrax, et al., 2009). Although, vitamin C is typically thought of as an antioxidant its accumulation in the presence of redox active compounds such as quinones leading to a futile non-enzymatic redox cycling. This redox cycling substantially increases the amount of intracellular ROS furthur impairing redox homeostasis in malignant cells that are already under high constitutive oxidative stress (Verrax, et al., 2009).

Vitamin K3 (also known as menadione) is a synthetic derivation of the naturally occurring vitamin K1 and K2 that can become alkylated into active isoprenylated menaquinones after absorbtion. Several studies have shown the anti-cancer activity of menadione both in vivo and in vitro. In addition, a synergistic effect was observed when
menadione was combined with chemotherapeutic agents. Vitamin K3/C combination has shown anticancer effects both *in vivo* and *in vitro* without the use of chemotherapy and radiation. Cancer cells seem to be more sensitive towards the cytotoxic effects of Vitamin K3/C mixture.

The synergistic anticancer effect of vitamin K₃/C mixture is likely explained by the redox-cycling that occurs between these compounds. This generates ROS and leads to an oxidative stress particularly deleterious for cancer cells since they exhibit a poor antioxidant status (Verrax, et al., 2008). Thus, Vitamin K₃/C mixture appeared to be selective for cancer cells. Indeed histopathological examination did not indicate any sign of toxicity in normal organs and tissues of vitamin K₃/C-treated mice.
Chapter 2: Results

Carcinoids are generally defined as slow-growing tumors which may not be diagnosed until the very last stages of the disease. Because of the indolent nature of these cancers, and considering the important role of mitochondria in metabolism, we hypothesized that carcinoid cell lines may contain lower number of mitochondria or dysfunctional mitochondria in comparison to more aggressive metabolically active cancer cell lines.

2.1 Carcinoid cell lines appear to contain fewer mitochondria in comparison to more aggressive breast cancer cell lines.

In order to determine mitochondrial density, we transiently transfected both the carcinoid (BON, CNDT2.5) and breast cancer (MCF7, MDA-MB231) cell lines with Ds-Red Mito, a mammalian expression vector containing a mitochondrial targeting sequence to label mitochondria. In addition, we used green-fluorescent Calcein AM dye to label the cytoplasm of transfected cells prior to imaging. Mitochondria and cytoplasm of cancer cells were visualized using wide-field fluorescence live cell imaging method. The intensity of red (Ds-Red Mito) and green (Calcein) fluorescence was measured using TILL VISION software. As shown in the representative images the majority of mitochondria in carcinoid cell lines appeared to distribute in a perinuclear fashion whereas breast cancer mitochondria appeared to distribute more evenly throughout the cytoplasm (Figure 2-1: A-D). The averaged ratio values of red to green signals are shown
in Figure 2-1E and indicated mitochondrial density in carcinoid and breast cancer cell lines. The ratio values were 0.1639 a.u.±0.02006 for BON, 0.05967 a.u.± 0.008419 for CNDT, 0.1936 a.u.±0.02323 for MCF7, and 0.5674 a.u.±0.1586 for MDA-MB231 cell lines.

Thus breast cancer cells, in particular the MDA cell line, exhibited greater ratio value compared to carcinoid cell lines. It is important to note that MDA cells are considered as one of the most aggressive and invasive form of breast cancer cells. Consistent with our initial prediction, these results suggested that carcinoid cells contained fewer mitochondria compared to breast cancer cells.

To further observe mitochondrial localization and distribution within cancer cells, we performed confocal microscopy in which multiple, in-focus optical slices were obtained at 1 µm intervals. Typically, we optically sectioned through the entire cell (usually 10-25 µm). These image stacks were collapsed as projected images for representation on the figures or used to construct three-dimensional animations. Representative images are shown in (Figure 2-2: A-D). These data largely corroborated our wide-field fluorescence findings that carcinoid cell lines had lower density and more restricted distribution of mitochondria than did breast cancer cells. Moreover, the higher resolution of confocal microscopy allowed for better visualization of morphology of individual or small clusters of mitochondria in cancer cells. Most of the mitochondria were rod shaped (BON, MCF-7) or more filamentous (CNDT2.5, MDA). One curious observation was that in BON and MCF-7 cells many of the individual mitochondria adopted a “doughnut-like” morphology. We have no explanation for these intriguing structures. In addition, volume reconstructions showed that BON cells exhibited a
“rounded” or “dome-like” structure. Although many mitochondria appeared to encircle the cell nucleus, a significant number of the mitochondria were distributed in the region of the cell we called “the footprint” in close proximity to the cell-glass interface. Volume reconstructions revealed that the other cell types investigated had a more flattened morphology. Similar to BON cells, CNDT2.5 cells also exhibited a preponderance of mitochondria in the footprint region. In contrast, MCF-7 and MDA-MB231 cells exhibited a more even distribution of mitochondria in the z-axis, but had fewer detectable mitochondria in their footprint regions.

2.2 Mitochondria in carcinoid cells exhibit a lower energy state compared to breast cancer cells.

In addition to a reduction in mitochondrial content, we hypothesized that the mitochondria of carcinoid cell lines may also be functionally compromised. Thus to address this possibility, we determined mitochondrial inner membrane potential ($\Psi_m$), an important indicator of mitochondrial function. Cancer cells were treated with JC-1, a vital semi-quantitative $\Psi_m$ indicator dye, and examined using fluorescent microscopy. JC-1 is positively charged and accumulates in the mitochondria due to the negative inner mitochondrial membrane potential ($\Psi_m = -180$ to $-220$ mV). Polarized $\Psi_m$ promotes JC-1 to accumulation and formation of red fluorescent aggregates. In contrast, under a low membrane potential, JC-1 does not accumulate, stays in monomeric form and fluoresces green. In addition JC-1 can redistribute to cytoplasm if mitochondria become depolarized. The red/green ratio therefore can indicate the membrane potential status.

As shown in Figure 2-3 B, JC-1 staining revealed that mitochondria of carcinoid cell lines possess a slightly lower membrane potential in comparison to breast cancer cell
The ratio values were 1.227 a.u. ± 0.167 for BON, 1.693 a.u. ± 0.1627 for CNDT2.5, 1.834 a.u. ± 0.2177 for MCF-7, and 2.145 a.u. ± 0.2894 for MDA-MB231 cell lines; (n=7-9) consistent with our hypothesis that carcinoid cell lines exhibit lower $\Psi_m$ than more metabolically active breast cancer cell lines. The observed diminished $\Psi_m$ may have implications for a role for mitochondria in responding to Ca$^{2+}$ entry and maintenance of Ca$^{2+}$ homeostasis in carcinoid cell lines because mitochondria is the driving force for mitochondrial Ca$^{2+}$ entry. Thus we chose the mitochondrial inhibitor FCCP (p-trifluoromethoxy carbonyl cyanide phenyl hydrazone), as a pharmacological tool to abrogate $\Psi_m$ and test whether mitochondria in carcinoid cells detect Ca$^{2+}$ entry or contribute to the response induced by a Ca$^{2+}$ challenge. As a prelude to this set of experiments we tested the effectiveness of FCCP, a protonophore that uncouples mitochondrial inner membrane potential and diminishes the driving force for mitochondrial Ca$^{2+}$ import. As shown in Figure 2-3 C, BON cells were stained with JC-1 and treated with FCCP prior to fluorescence imaging. We observed a significant diminishment in mitochondrial membrane potential following FCCP treatment validating our use of FCCP as a means to diminish mitochondrial Ca$^{2+}$ import and assess the role of mitochondria in modulating Ca$^{2+}$ dynamics in cancer cell lines.

2.3 Mitochondrial inhibition alters the response to Ca$^{2+}$ entry.

In many cell types, mitochondria are known to respond to and influence the entry of Ca$^{2+}$ through plasma membrane ion channels including glutamate receptors, VOCCs and SOCCs (Hoth et al., 1997). Previous work (Giovannucci et al. 1999) demonstrated that mitochondrial inhibition enhanced Ca$^{2+}$ dependent Ca$^{2+}$ channel inactivation in bovine chromaffin cells (a neuroendocrine cell model). Thus, we initially investigated
whether mitochondrial inhibition would also alter VOCCs in neuroendocrine carcinoid cell lines. However, we quickly abandoned this line of inquiry for the following reasons: 1) it became apparent from our study that FCCP did not significantly alter the Ca\(^{2+}\) signals evoked by VOCC activation in BON cells, and 2) none of the other cell lines tested expressed functional high voltage activated Ca\(^{2+}\) channels. Thus we took an alternative approach to induce Ca\(^{2+}\) entry.

Others in our lab showed that mitochondrial inhibitors diminished SOCE in HEK293 cells likely due to Ca\(^{2+}\) dependent SOCC inactivation (Mignen et. al, 2006). Moreover, our lab recently showed that carcinoid cell lines exhibit functional SOCE (Arunachalam et. al, 2010).

In this current set of experiments, we showed that all four of the cell lines used in the current study exhibit SOCE in response to ER Ca\(^{2+}\) store depletion (Figure 2-4: A-D). To examine whether mitochondria respond to Ca\(^{2+}\) influx through store-operated Ca\(^{2+}\) channels (SOCC) in carcinoid and breast cancer cell lines, we loaded cancer cells with Fura-2, a high affinity Ca\(^{2+}\) indicator and visualized Ca\(^{2+}\) dynamics using live cell imaging method. To stimulate SOCE, cells were treated with Ca\(^{2+}\) free physiological saline containing 10 µM cyclopiazonic acid (CPA), a SERCA pump inhibitor. CPA treatment was characterized by a slow transient rise in cytosolic Ca\(^{2+}\) most likely reflecting the leak of Ca\(^{2+}\) from ER and subsequent extrusion by PMCA.

SOCE was then assayed by application of saline solution containing 5mM Ca\(^{2+}\) with or without addition of FCCP. FCCP increases the proton permeability of inner mitochondrial membrane dissipating the membrane potential which leads to reduced mitochondrial Ca\(^{2+}\) import. The mitochondrial ATPase blocker oligomycin was always
included in the experiments to prevent ATPase reversal and consumption of cytosolic ATP content. We then measured the rate and the peak amplitude of store-operated Ca\(^{2+}\) entry under control and FCCP treated conditions.

To determine the amplitude, we measured the peak value of cytoplasmic Ca\(^{2+}\) rise under both control and treated conditions. The averaged peak amplitudes were as followed: 628.7 r.u.±42.72 for BON, 959.5 r.u.±64.84 for CNDT2.5, 238.2 r.u.±25.08 for MCF-7, and 1188 r.u.±138.0 for MDA-MB231 cell lines. As shown in Figure 2-4 E, the amplitude was not significantly affected. In contrast, we observed significant differences in the rate of Ca\(^{2+}\) rise between control and treated carcinoid cell lines but not for breast cancer cell lines.

The rate of Ca\(^{2+}\) rise induced by SOCE was determined by calculating the half time to reach peak change in the Ca\(^{2+}\) signal (Fig 2-4 F). Under control conditions the averaged half time to peak were respectively as followed: 72.52 s±4.105 for BON, 193.7s±22.52 for CNDT2.5, 107.1s±8.605 for MCF-7, and 70.02s±7.799 for MDA-MB231 cell lines (n=12-39).

These data suggested that mitochondria of carcinoid cells were important in regulating Ca\(^{2+}\) entry in carcinoid cell lines and that a subpopulation of mitochondria may be in close proximity to entry sites. To test this possibility, we performed TIRF (total internal reflection) microscopy. This method allowed us to observe the distribution of mitochondria located only beneath the plasma membrane. TIRF microscope uses an evanescent wave that excites surface delimited fluorescent structures between 100-200 nm from the plasma membrane-glass interface. Evanescent waves decay exponentially with distance from the interface at which they are formed and thus fluorescent signals
exhibit superior z-axis resolution. In this set of experiments, cancer cells were transfected with Ds-Red Mito and loaded with Calcein AM as described previously. For each designated cell line, both wide-field and TIRF images were obtained using the Slide Book software and a laser-based Olympus 3-color TIRF system coupled to an IX-81 microscope equipped with a 60X high NA (1.4) objective. As shown in Figure 2-5: A-B acquired images revealed that BON and CNDT cell lines appear to contain mitochondria in very close proximity to the plasma membrane. In contrast, MCF-7 and MDA-MB231 cell lines did not show sub plasma membrane mitochondria (data not shown). These observations were consistent with our previous data and overall suggested that mitochondria are important regulators of SOCE in carcinoid cell lines and critical for the maintenance of Ca\(^{2+}\) homeostasis.

These combined observations placed carcinoid mitochondria as a potential target for inducing Ca\(^{2+}\) dysregulation and toxicity. However, a major obstacle in this endeavor is how to selectively inhibit mitochondrial function and Ca\(^{2+}\) homeostasis in cancer cells but not in non-transformed cells.

2.4 The toxic effect of Vit K\(_3\)/C mixture on carcinoid and breast cancer cells viability

_Verrax et.al,2009_ had previously shown that a combination of vitamin C and a quinone compound such as vitamin K\(_3\) will undergo a redox cycling that leads to overproduction of reactive oxygen species (ROS). Importantly, cancer cells typically overexpress vitamin C transporters and thus accumulate high amounts of ascorbate. Thus, when treated with elevated levels of these nutrients, the resulting oxidative damage can rapidly kill cancer cells but leave normal cells unaffected. Since mitochondria are known
as major sites of ROS production and ROS damage, they once again present as potential targets for this anti-cancer treatment. To assess the effect of Vit K₃/C treatment on cancer cell growth, we performed CyQuant NF cell proliferation assay on carcinoid and breast cancer cell lines. Using a DNA-binding dye in combined with a plasma membrane permeabilization reagent, CyQuant assay provides a fast and sensitive way for counting cells and to measure cell proliferation. Cells plated on a 96-well microplate were first treated with Vit K₃/C mixture and incubated for 24 hours at 37°C. The CyQuant assay was performed following a 30 minute incubation and the cell number was measured using a FLUO-star OPTIMA microplate reader. As shown in Figure 2-6, carcinoid cell lines in contrast to our predictions exhibited more resistance to Vit K₃/C toxicity compared to breast cancer cell lines. Data demonstrate the percentage of cells surviving after 24 hours of treatment compared to control. For instance, there was a 50% survival rate in BON cells following Vit K₃/C treatment compared to only a 17% survival rate in MDA-MB231 cell line. The percent values were 133.7± 16.10 and 53.18± 17.02 for CNDT and BON respectively (n=3). This data was in contrast to our initial prediction which had put carcinoids in a greater risk for cell death given lower Ψₘ values and reduced number of their mitochondria. One possibility for this discrepancy is that ROS were produced at lower levels in carcinoid cell lines.

To further investigate these findings, we decided to assess ROS production in carcinoid and breast cancer cells with and without Vit K₃/C treatment.

2.5 Measuring ROS production in carcinoid and breast cancer cells

Initially, we chose to measure the production level of hydrogen peroxide because H₂O₂ has been implicated as the dominant toxic product produced by Vit K₃/C oxidant
therapy (Verrax et. al, 2009). Cancer cells were loaded with CM-H2DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate) a cell-permeant indicator dye to label H₂O₂ and treated with Vit K₃/C mixture for 45 minutes. CM-H2DCFDA passively diffuses into cells where its acetate groups are cleaved by intracellular esterases allowing the chloromethyl groups to react with intracellular thiols. Oxidation of CM-H2DCFDA yields a fluorescent adduct dichlorofluorescein (DCF) that is trapped inside the cell. The emitted fluorescence from CM-H2DCFDA is proportional to intracellular concentration of H₂O₂. Using a wide-field fluorescence microscope, H₂O₂ production was detected at an excitation wavelength of 492 nm and emission of 517 nm (Figure 2-7A). The average quantified value of fluorescence intensity is also indicated in Figure 2-7 B.

These experiments revealed that H₂O₂ production was enhanced in all four cell lines following Vit K₃/C treatment. Where H₂O₂ production is thought to be the pathway mediating cell death, increased levels of H₂O₂ were relatively well tolerated by carcinoid cell lines in contrast to previous studies. Although, MCF7 and MDA-MB231 cell lines showed the highest increase in H₂O₂ production, these findings suggested that enhanced production of H₂O₂ upon Vit K₃/C treatment is unlikely to be the underlying cause of differential cell toxicity.

In addition to H₂O₂, superoxide anion is also known to be a potent and damaging oxidative molecule. Moreover, mitochondria are considered as major sites of superoxide production and toxicity. Thus, we decided to measure superoxide anion production in response to Vit K₃/C treatment. In these experiments, cancer cells were labeled with MitoSOX Red, a novel fluorogenic, highly selective mitochondrial superoxide indicator, treated with Vit K₃/C for 45 minutes and followed by live cell imaging methods.
MitoSOX Red is cell permeant and selectively targeted to mitochondria. Once in the mitochondria, MitoSOX can be oxidized by superoxide and fluoresces red.

Images revealed that super oxide production was greatly increased in breast cancer cell lines compared to carcinoid cell lines (Figure 8B). Moreover, there was a correlation between mitochondrial superoxide and diminishment of cell number.

The lower mitochondrial superoxide levels in carcinoid cells compared to breast cancer cells suggested that the mitochondrial function in carcinoid cells was better preserved. To test this idea, we used JC-1 measurements prior to and following Vit K₃/C treatment.

As mentioned earlier, the membrane potential of mitochondria is a major parameter of mitochondrial function. Cancer cells were labeled with JC-1, treated with Vit K₃/C mixture, and visualized using a wide-field fluorescence microscope. As indicated in Fig 2-9, Ψₘ was reduced in breast cancer cells and less so in carcinoid cells following treatment. Ratio values for control and treated cell lines are: 0.5131 for BON, n=8, P-value= 0.0177, 0.3193 for CNDT2.5, n=7, P-value= 0.1223, 1.154 for MCF-7, n=9, P-value= 0.0024, and 1.012 for MDA-MB231, n=8, P-value= 0.0120.

Thus, these novel data indicated that rather than H₂O₂, it is likely that superoxide mediated cell toxicity in part via mitochondrial damage in response to Vit K₃/C treatment.
Chapter 3: Discussion

As rare, slow-growing gastrointestinal neuroendocrine tumors, carcinoids have imparted numerous diagnostic and therapeutic challenges over the years and in most cases, the cancer is not diagnosed until the very last stages of the disease (K. Oberg, 1996). Moreover there are currently few effective treatment options available for patients. Thus there is a need to develop new strategies to treat this disease.

Previous work in our laboratory has shown that carcinoid cell lines express a variety of VOCCs and SOCCs and that mitochondria are essential in regulating Ca\(^{2+}\) entry through these plasma membrane channels. We have also shown that inhibiting store-operated Ca\(^{2+}\) entry antagonizes mitochondrial Ca\(^{2+}\) import which in turn affects local Ca\(^{2+}\) clearance (Giovannucci et al, 1999). Therefore, in addition to their central role in metabolism, mitochondria are key regulators of Ca\(^{2+}\) homeostasis. Moreover, defective mitochondria are often associated with many different disorders including cancer (Carew & Huang, 2002). These observations point to mitochondria as possible therapeutic targets for an anti-cancer treatment in carcinoids.

In the current study, we applied an oxidative anti-cancer treatment regiment to specifically target carcinoid cancer cell mitochondria that should limit the treatment effects to cancer cells only. As a first step we decided to assess mitochondrial mass and functional status in BON and CNDT cells, a foregut and a midgut carcinoid cell line respectively. We also chose MCF-7 and MDA-MB231 breast cancer cells which
represent more aggressive cancer cell lines, and are presumably more metabolically active. Our simple idea was to test the hypothesis that carcinoid mitochondria might have less capacity than normal or other cancer cells to maintain Ca^{2+} homeostasis in the presence of a mitotoxic challenge, and thus expose carcinoid cancer cells to Ca^{2+} overload induced cell death. We measured mitochondrial density and membrane potential using wide-field fluorescence microscopy. Our results indicate that carcinoid cells contain fewer, less energized mitochondria compared to breast cancer cells.

To obtain higher resolution images of mitochondria, we performed confocal microscopy which revealed intriguing differences in distribution, shape and size between mitochondria of carcinoid and breast cancer cell lines (Fig. 2A-D). For instance, mitochondria were distributed in carcinoid cell lines primarily around the nucleus and in the footprint region whereas in breast cancer cell lines they were more evenly spread throughout the cytoplasm. One speculation is that because carcinoid cell lines have fewer mitochondria, the remaining mitochondria are distributed to essential sites for local ATP production or Ca^{2+} homeostasis. In addition, there were differences in the morphology of mitochondria among the cell lines. Mitochondria in BON and MCF7 cell lines were mostly rod shaped and some also exhibited a “doughnut-like” structure. However, in the MDA-MB231 and CNDt2.5 cell lines, mitochondria were more filamentous. The morphology of mitochondria is regulated by continuous fusion and fission events that are essential for maintaining a normal mitochondrial function. Often when mitochondria are under stress they adopt a fragmented and rounded state. Moreover, it has been suggested that mitochondrial morphology can impact mitochondrial function and cell metabolism (Grandemange, Herzig, & Martinou, 2009).
However, different cell types have mitochondria that come in a wide variety of shapes and can undergo dynamic changes in their morphology. Because we had no knowledge of the typical morphology in non transformed cells from which carcinoid cells arise, this line of investigation was not pursued any further in the current study.

Using Ca$^{2+}$ imaging techniques, we also examined the role mitochondria may play in regulating SOCE in both carcinoid and breast cancer cells. Following mitochondrial poisoning, the rates of Ca$^{2+}$ influx was significantly enhanced in carcinoids. This indicated that mitochondria normally import Ca$^{2+}$ that enters through SOCCs. Moreover, because mitochondria are typically low affinity Ca$^{2+}$ buffering component in cells; this suggested a close association between mitochondria and Ca$^{2+}$ entry sites.

Using TIRF (total internal reflection fluorescence) microscopy, we obtained images that revealed that although the majority of mitochondria resided around the nucleus in carcinoid cell lines, a significant number of mitochondria were present at the footprint area of the cell. We did not observe such mitochondrial distribution in breast cancer cells, consistent with our finding that breast cancer cells have fewer mitochondria near the predicted sites of Ca$^{2+}$ entry and thus do not sense or modulate Ca$^{2+}$ entry through SOCCs but are effective at responding to global (volume averaged) Ca$^{2+}$ signals.

The above mentioned characteristics of mitochondria in carcinoid cancer cells implied both a more compromised status in these cells and a more critical role in Ca$^{2+}$ homeostasis making the mitochondria all the more appealing as potential targets for anti-cancer treatment regimens.

Previous studies by Verrax et al. have shown that a combination of vitamin C and a quinone (vitamin K$_3$) undergoing a redox cycling in TLT cells leads to over production
of ROS, an oxidative stress and can rapidly kill cancer cells. Cancer cells generally show a poor antioxidant status and accumulate vitamin C. Most cells transport vitamin C through glucose transporters which are upregulated in a majority of cancers (Verrax et al. 2009).

Based on our observation regarding the role of mitochondria in carcinoid cell lines, and the fact that mitochondria are not only major sites for the production of ROS but targets for the toxic effects of ROS, we predicted carcinoid cells would be less capable of withstanding oxidant challenge and would exhibit Ca^{2+} overload and reduced survival compared to other cell lines. To test if carcinoid cell lines were indeed more susceptible to oxidative stress, we targeted the carcinoid and breast cancer cells by applying an oxidative anti-cancer regimen comprising of vitamin K_3 and vitamin C. After treating both the carcinoid and breast cancer cells with a vitamin K_3 and vitamin C (Vit K_3/C) mixture, we performed a CyQuant cell proliferation assay. Contrary to our initial prediction where we expected carcinoid cells to be more susceptible, they surprisingly exhibited more resistance to Vit K_3/C treatment as compared to the breast cancer cells. To further investigate this observation, we measured the production of two major ROS species: hydrogen peroxide (H_2O_2) and the superoxide anion. Upon treatment, H_2O_2 levels were enhanced in all four cell lines, however, superoxide production was significantly increased only in breast cancer cells. This suggested, in contrast to findings by others, that the dominant cytotoxic ROS was superoxide rather than peroxide.

Although the production of H_2O_2 has been reported as the causative pathway leading to cell toxicity and death (Verrax et al, 2008; Verrax et al, 2009), our growth
assay results appear to point to the superoxide anion as the responsible species in cancer cell death.

Several possibilities could account for the fact that the oxidative treatment is less effective for carcinoid cancer cells compared to breast cancer cells. Depending on their site of origin, carcinoids have the ability to produce large amounts of serotonin. Serotonin is derived from tryptophan and can function as a neurotransmitter, hormone and mitogenic factor mediating a variety of physiological functions including growth and differentiation (Zuetenhorst & Taal, 2005). Previous studies have suggested that serotonin may possess anti-oxidant properties. Both BON cells and CNDT2.5 cells produce large amounts of serotonin and thus this supply of serotonin or its precursor tryptophan may possibly protect these tumors against oxidative stress induced by anti-cancer treatments. One possible way to test this hypothesis is to pharmacologically inhibit serotonin production and then determine cell viability following Vit K\textsubscript{3}/C treatment.

Another explanation for carcinoids resistance to Vit K\textsubscript{3}/C treatment could be that differences exist within anti-oxidant system of carcinoid and breast cancer cells. The intracellular ROS homeostasis is under the tight control of enzymatic anti-oxidant proteins such as superoxide dismutase (SOD), catalase and GSH peroxidase) activities and/or non-enzymatic compounds (ascorbate, glutathione). For example, superoxide dismutase (the enzyme which converts superoxide to hydrogen peroxide) may be more active in breast cancer cells compared to carcinoiod cells.
In addition, although carcinoids favor glycolysis over oxidative phosphorylation for energy production, they may take up Vit K$_3$/C less effectively than the more metabolically active breast cancer cell lines. Although we have not directly measured this possibility, we think it is unlikely because carcinoid line produce high amounts of peroxide in response to Vit K$_3$/C treatment.

It is important to note that the use of Vit K$_3$/C as a treatment for prostate cancer is in phase II clinical trials and a patent has been awarded for a drug combination (Apotone) that provides doses of these nutrients comparable to those we have used in the current study (Tareen, et al., 2008). However, our data indicate that the oxidative treatment regimen as proposed by Verrax, et al., 2009 may not be effective for all cancers. Much further study will be necessary to determine if this treatment approach alone or in combination with other therapies will be useful as a strategy to combat carcinoid cancer in patients.
Chapter 4: Conclusion

Based on our data we conclude that:

1. Carcinoid cell lines appeared to contain fewer mitochondria compared to breast cancer cell lines
2. Mitochondria in carcinoid cells appeared more restricted (mainly localized to the footprint region) whereas in breast cancer cells we observed a more even distribution of mitochondria throughout the cytoplasm.
3. Carcinoid cell mitochondria appear to be less energized compared to mitochondria of breast cancer cells.
4. A sub population of mitochondria in carcinoid cancer cell lines may reside in close proximity to the plasma membrane suggesting that mitochondria are important in regulation of Ca\(^{2+}\) entry.
5. Dysregulation of mitochondria appears to be more effective at altering Ca\(^{2+}\) signaling in carcinoid cell lines than in breast cancer cell lines.
6. Carcinoid cells exhibited more resistance towards oxidative stress induced by Vit K\(_3\)/C treatment compared to breast cancer cell lines.
7. Hydrogen peroxide levels were significantly increased in both carcinoid and breast cancer cell lines after VitK\(_3\)/C treatment.
8. Superoxide levels were significantly increased only in breast cancer cell lines after VitK\(_3\)/C treatment.
9. The increased level of superoxide correlates with cell death.

10. Mitochondrial membrane potential was reduced in both carcinoid and breast cancer cell lines after VitK$_3$/C treatment

11. Foregut carcinoid cells mitochondria may be susceptible to Vit K$_3$/C-based oxidant therapy.
Chapter 5: Materials & Methods

Cell culture. In the current study, we used human foregut (BON) and midgut (CNDT2.5) carcinoid cell lines along with MCF-7 and MDA-MB231 breast cancer cell lines. BON, MCF-7 and MDA-MB231 cell lines were grown in Dulbecco’s Modified Essential Medium (DMEM) supplemented with 10% FBS. CNDT2.5 cell line was maintained in DMEM supplemented with 10% FBS and 1% sodium pyruvate and 1% HEPES. Penicillin/streptomycin Gibco #15410 (1%) was added to all media. All the cell lines were maintained at 37 °C in a humidified incubator set at 5% CO2. The cell lines were harvested following brief treatment with Trypsin/EDTA (0.25%). All cell lines were passaged at the ratios recommended by provider. BON cells were provided by Dr. Kjell Oberg, Uppsala sweden.

Live cell imaging. For live cell imaging experiments, cells were grown on clean glass coverslips that were used to form the bottom of a recording chamber.

Confocal microscopy. The images were obtained using a Ziess 510 Meta laser-scanning confocal microscope equipped with an Axiovert 200 MOT microscope with a 63X/NA 1.4 Plan-Apo oil-immersion objective.

Calcium measurements. Changes in cytosolic Ca^{2+} levels were monitored using fura-2 fluorescence method. For loading with fura-2 AM, cells were incubated with physiological saline solution (containing 140 mM NaCl, 5 mM KCl, 2.2 mM CaCl2, and...
1 mM MgCl₂, and 10 mM HEPES, and 5 mM glucose, pH 7.35) containing 2 μM fura-2 AM for 30-40 min at 25°C. Following loading protocol, cells were mounted on the stage of an Olympus IX-71 microscope equipped with a 40X oil immersion objective (NA = 1.4). An air pressure driven device was used to exchange physiological saline or solutions containing agonists or antagonists by local application through a glass capillary placed at the edge of the field of view. Cells were alternately illuminated at 340 and 380 nm light focused using a fiber optic guide and epifluorescence condenser onto the image plane by dichroic mirror (Semrock) using a monochromator-based Polychrome IV imaging system (TILL Photonics) and emission was detected using 510 nm ± 25 nm band pass filter (Semrock) and IMAGO QE camera. Changes in intracellular calcium were represented by and expressed as the ratio of fura-2 fluorescence (F340/F380).

Mitochondrial membrane potential (ψₘ) measurements. Cells (5 X 10⁴ cells/ml) were labeled with 2 ug/ml JC-1 in physiological saline solution and incubated for 30 minutes at 25°C. We used FCCP at a 3uM concentration to depolarize ψₘ.

H₂O₂ measurements. To measure H₂O₂ production, cells were incubated with physiological saline solution (containing 140 mM NaCl, 5 mM KCl, 2.2 mM CaCl₂, and 1 mM MgCl₂, and 10 mM HEPES, and 5 mM glucose, pH 7.35) containing 2 μM CM-H₂DCFDA (5-(and-6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate, acetyl ester) for 30 min at 37°C. CM-H₂DCFDA passively diffuses into cells where its acetate groups are cleaved by intracellular estrases allowing the chloromethyl groups to react with intacellular thiols including glutathione. Oxidation yields a fluorescent adduct that is trapped in the cell. The fluorescence emitted by CM-H₂DCFDA is proportional to its interaction with intracellular ROS. (Ex/Em: 492/517 nm)
Superoxide measurements. Superoxide production was measured using MitoSOX Red dye indicator. Cells were loaded with MitoSOX and incubated in physiological saline solution (containing 140 mM NaCl, 5 mM KCl, 2.2 mM CaCl₂, and 1 mM MgCl₂, and 10 mM HEPES, and 5 mM glucose, pH 7.35) containing 10 uM MitoSOX Red for 30 min at 37°C. MitoSOX Red is a live-cell permeant and is selectively targeted to the mitochondria. Once in the mitochondria, the dye is oxidized by superoxide and exhibits red fluorescence (Ex/Em: 510/580 nm)

Vit K₃/C treatment. Cells were treated with Vit K₃/C and incubated for 45 minutes prior to imaging for MitoSOX and CH-H2DCFDA. For JC-1 imaging we treated the cells for 24 hours.

Vit K₃/C mixture: 1uM vitamin C in combined with 10uM vitamin K (1:100) in physiological saline solution

CyQuant cell proliferation assay. Cells were plated in 48-well plates at a density of 10,000 cells/ml in complete medium (DMEM supplemented with 10% FBS and Penicillin/streptomycin (1%)) and incubated for 24 hours at 37°C. cells were then treated with Vit K₃/C and incubated for 24 hours at 37°C prior to measurement by FLUO star OPTIMA microplate reader.


