Calcium influx and release controls neuroendocrine cell secretion and excitability

By

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A thesis submitted to the Department of Physiology
in conformity with the requirements for
the degree of Master of Science

Queen’s University
Kingston, Ontario, Canada
September 2009

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Abstract

Ca\(^{2+}\) dynamics affect many critical cellular processes. In the bag cell neurons of *Aplysia californica*, intracellular Ca\(^{2+}\) is elevated during a prolonged period of firing known as the afterdischarge. This consists of a fast and slow phase of firing, which triggers peptide secretion and culminates in egg-laying. The present study examines how Ca\(^{2+}\) influx and release shape neurosecretion and membrane activity. Using capacitance tracking as an index of secretion, a 5 Hz, 1 min train, to mimic the fast phase, induced a clear elevation in the membrane surface area of cultured bag cell neurons. The capacitance change was abolished by replacing external Ca\(^{2+}\) with Ba\(^{2+}\) or addition of the Ca\(^{2+}\) channel blocker, Ni\(^{2+}\). Additionally, the response was reduced by either strong buffering of intracellular Ca\(^{2+}\) or pretreatment with N-ethylmaleimide, an alkylating agent that disrupts vesicular transport. Depleting mitochondrial Ca\(^{2+}\) with the protonophore, carbonyl cyanide-\(p\)-trifluoromethoxyphenyl-hydrazone (FCCP), also elevated capacitance, while depleting endoplasmic reticulum Ca\(^{2+}\) with the Ca\(^{2+}\)-ATPase inhibitor, cyclopiazonic acid, did not. Similarly, FCCP alone depolarized bag cell neurons. In a concentration-dependent manner, FCCP elicited an inward current that was insensitive to Ni\(^{2+}\), associated with an increase in conductance, and a linear current/voltage relationship that reversed around -40 mV. Removal of extracellular Ca\(^{2+}\) reduced the current and left-shifted the reversal, consistent with opening a Ca\(^{3+}\)-permeable, voltage-independent, non-selective cation channel. The current was decreased when intracellular Ca\(^{2+}\) was strongly buffered, while fura-imaging demonstrated that FCCP elevated intracellular Ca\(^{2+}\) with a similar time course, suggesting a dependence on intracellular Ca\(^{2+}\). Although both oligomycin A and bafilomycin A, inhibitors of mitochondrial ATP synthetase and V-type H\(^{+}\)-ATPase, respectively, gradually increased Ca\(^{2+}\), neither produced a current. The FCCP-induced Ca\(^{2+}\) elevation and the current were also diminished by disabling the mitochondrial permeability transition pore with N-ethylmaleimide. The data suggests that a cation current is preferentially gated by Ca\(^{2+}\) released from the
mitochondria, rather than disruption of ATP production. This current could provide depolarizing drive for the afterdischarge. While Ca\textsuperscript{2+} entry appears to be responsible for initiating neurosecretion, mitochondrial Ca\textsuperscript{2+} may support prolonged peptide release during and subsequent to the afterdischarge.
Contributions

The following thesis expands upon preliminary discoveries made in our laboratory by Ms. Julia Geiger (MSc graduate) and Dr. Neil Magoski regarding Ca\(^{2+}\) influx and release in *Aplysia* bag cell neurons. Dr. Magoski trained me in whole-cell and sharp-electrode recording, as well as capacitance tracking and fluorescence imaging. Dr. Magoski and I discussed results, observations, experimental procedures and relevant literature on a regular basis.

I performed all of the data recordings and analysis contained within this thesis, with the exception of the fluorescent imaging of bag cell neurons treated with CCCP, which was done by Ms. Geiger. I mostly prepared my own solutions and drugs, with occasional assistance from Ms. Shannon Smith, our laboratory technician. Ms. Smith also provided care for the animals. I cultured most of the cells that were intended for my own use; however, I shared the duties involved with animal dissection with other members of the lab.

I prepared this complete thesis with necessary editing and constructive criticism from Dr. Magoski. The work in this thesis will be summarized into two manuscripts, written together with Dr. Magoski. One paper will be based on the data presented here concerning the activation of a Ca\(^{2+}\)-dependent non-selective cation channel by mitochondrial Ca\(^{2+}\), while the other paper will include the data regarding secretion elicited by Ca\(^{2+}\) influx during the fast phase of the afterdischarge.
Acknowledgements

I would like to sincerely thank my supervisor, who is truly a wonderful teacher, Dr. Neil Magoski. Throughout my degree, he has provided me with thorough, patient and helpful guidance. The enthusiasm he has for his research is contagious and I could always expect that he would be just as excited as I was when I would run over to his office with any kind of breakthrough. Most of all, I count myself lucky to have a supervisor who so genuinely cares about the well-being of his students.

My thanks go to Shannon Smith who, whenever I felt overwhelmed about my work, would remind me that there is always tomorrow; Julia Geiger for her warmth and encouragement; Tim St. Amand who never failed to make me smile; and Sean White for knowing when to throw on some “Mr. Pitiful.” I would especially like to thank Alan Tam for his incredible friendship. His constant thoughtfulness and selflessness is remarkable and I am so grateful to have worked with him. I would also like to thank him for answering all of my questions that I was too embarrassed to ask Neil!

I am grateful to Dr. Al Ferguson and his lab for giving me my first experience in research. I have been fortunate to have had many outstanding teachers in physiology and neuroscience along the way and was inspired to carry on in the field because of them. In addition, I would like to thank the Department of Physiology, Queen’s University and Ontario Graduate Scholarships in Science and Technology (OGSST) for generously providing the funding to support my research interests over the past two years.

I am deeply thankful to my family and friends for their endless love and support, and for being my personal cheering section while I was writing my thesis.

My dearest Scott, I thank you with all of my heart. You were my strength when I needed it the most. I was able to make it this far because you were by my side every step of the way. You are truly my best friend.
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<th>Definition</th>
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<tbody>
<tr>
<td>$\alpha_{1C}$</td>
<td>alpha 1C subunit of calcium channel</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>$\text{Ba}^{2+}$</td>
<td>barium ion</td>
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<tr>
<td>BAPTA</td>
<td>1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>$\text{Ca}^{2+}$</td>
<td>calcium ion</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>calmodulin kinase II</td>
</tr>
<tr>
<td>CaRE</td>
<td>$\text{Ca}^{2+}$ response element</td>
</tr>
<tr>
<td>CaXΔ</td>
<td>calcium exchanger</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide 3-chlorophenylhydrazone</td>
</tr>
<tr>
<td>cfASW</td>
<td>calcium free artificial sea water</td>
</tr>
<tr>
<td>CICR</td>
<td>calcium-induced calcium release</td>
</tr>
<tr>
<td>$C_m$</td>
<td>membrane capacitance</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclopiazonic acid</td>
</tr>
<tr>
<td>CRE</td>
<td>cyclic adenosine monophosphate (cAMP) response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic adenosine monophosphate (cAMP) responsive element binding</td>
</tr>
<tr>
<td>$\text{Cs}^+$</td>
<td>cesium ion</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>half-maximal effective concentration</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol bis (aminoethylether) tetraacetic acid</td>
</tr>
<tr>
<td>ELH</td>
<td>egg-laying hormone</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
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<tr>
<td>FCCP</td>
<td>carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone</td>
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<tr>
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<td>G-protein</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid</td>
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<tr>
<td>$H^+$</td>
<td>hydrogen ion</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz (1/s)</td>
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<tr>
<td>$I_{\text{Ca}^{2+}}$</td>
<td>voltage-gated calcium current</td>
</tr>
<tr>
<td>$I_{\text{cat}}$</td>
<td>cation current</td>
</tr>
<tr>
<td>$I_i$</td>
<td>initial current</td>
</tr>
<tr>
<td>$I_{ss}$</td>
<td>steady-state current</td>
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<tr>
<td>IP$_3$</td>
<td>inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>$K^+$</td>
<td>potassium ion</td>
</tr>
<tr>
<td>kPa</td>
<td>kilopascal</td>
</tr>
<tr>
<td>MΩ</td>
<td>megaohms</td>
</tr>
<tr>
<td>MCU</td>
<td>mitochondrial $\text{Ca}^{2+}$ uniporter</td>
</tr>
<tr>
<td>MEF2</td>
<td>myocyte enhancer factor-2</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>magnesium ion</td>
</tr>
<tr>
<td>Mit</td>
<td>mitochondria</td>
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</table>
MPTP  mitochondrial permeability transition pore
ms    millisecond
mV    millivolt
n    sample number x
NA   numerical aperture
Na+  sodium ion
nA   nanoampere
nF   nanofarad
nASW  normal artificial salt water
Ni2+  nickel ion
NEM  N-ethylmaleimide
NMAD  N-methyl-D-aspartic acid D-glucamine
NSF  N-ethylmaleimide-sensitive factor
pA   picoampere
Per1  Period1 gene
pF   picofarad
pH   negative logarithm of hydrogen ion concentration
PKA  protein kinase A
PKC  protein kinase C
pS   picoisien
Ra   access resistance
Rm   membrane resistance
ROI  region of interest
RyR  ryanodine receptor
SE   sharp-electrode
SERCA  sarcoplasmic-endoplasmic reticulum Ca2+-ATPases
SKF-96365  1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride
SNAP 25 soluble NSF attachment protein 25 kD
SNARE soluble N-ethylmaleimide-sensitive factor attachment protein receptors
τ   time constant
tcASW  tissue culture artificial salt water
TEA  tetraethyl ammonium
TPP  tetraphenylphosphonium
TRP  transient receptor potential
TRPC  transient receptor potential canonical subfamily
TRPM  transient receptor potential melastatin subfamily
UV   ultraviolet
Vs   magnitude of the voltage step
WC   whole-cell
Chapter 1: Introduction

Controlling fundamental behaviours

The release of neuropeptides into the circulation by the neuroendocrine system is responsible for the initiation of essential behaviours that are common to all complex organisms, such as eating, drinking, sleeping, mating and reproduction (Silverthorn, 2004). Behavioural sequences controlled by the neuroendocrine system can be quite complex, especially since neuropeptides have the ability to modulate the activity of many neurons. Konrad Lorenz was the first to suggest that there are instinctive behavioural sequences, called fixed action patterns, which are programmed by specific neural networks and will run to completion once triggered by a stimulus (reviewed in Halliday, 1998). For example, the escape response of the sea slug, Tritonia, is a fixed action pattern, in which the bursting activity of a specific group of neurons stimulates a defined sequence of behaviours, including withdrawal of the animal followed by alternating contractions of dorsal and ventral muscles resulting in prolonged swimming (Willows & Hoyle, 1969). The mating behaviour of Drosophila is another fixed action pattern that is controlled by a specific neural network (Hotta & Benzer, 1976).

Such complex behavioural patterns can be produced by the stimulation of a few neurons, or even single neurons. Wiersma and Ikeda (1964) observed that the rhythmic beating of crayfish swimmerets is caused by the stimulation of single interneurons. Thereafter, individual or small sets of neurons capable of generating specific complex behavioural responses became known as command neurons (Wiersma & Ikeda, 1964; Frost & Katz, 1996; Edwards et al. 1999). The command neuron was more strictly defined by Kupfermann and Weiss (1978) as a neuron whose activity is necessary and sufficient for the initiation of a specific behaviour.

Changes to intracellular Ca$^{2+}$

The triggering of behaviour by a command neuron requires a change in the activity and output of the cell. This almost always involves altering intracellular Ca$^{2+}$, a ubiquitous cellular
signal. Ca\textsuperscript{2+} is a unique and fundamental signal because it translates electrical activity into biochemical activity (Hille, 2001). Ca\textsuperscript{2+} enters the cell, primarily through voltage-gated Ca\textsuperscript{2+} channels, where it serves as a second messenger triggering a variety of intracellular responses. Initially discovered in crustacean muscle fibres (Fatt & Katz, 1953), voltage-gated Ca\textsuperscript{2+} channels have since been identified in all excitable cells (Hagiwara, 1983). There are two distinct classes of Ca\textsuperscript{2+} currents based on their activation properties: low-voltage activated (Akaike, 1991; Huguenard, 1996) and high-voltage activated (Tsien et al. 1988; Bean 1989; Carbone & Swandulla, 1989; Kostyuk, 1989). The tiny and transient T-type Ca\textsuperscript{2+} channels are classified as low-voltage activated since they are activated at potentials slightly negative to the resting membrane potential (Huguenard, 1996). On the other hand, significant depolarization is required to open high-voltage activated channels (Huguenard, 1996; Ertel et al. 2000; Hille, 2001). The long-lasting L-type Ca\textsuperscript{2+} channels, often involved with peptide and hormone release, activate at membrane potentials higher than -10 mV (Nowycky et al. 1985). N-type Ca\textsuperscript{2+} channels require considerable depolarization for activation, yet very negative potentials to remove inactivation (Nowycky et al. 1985). The N-type, as well as the larger P/Q-type, Ca\textsuperscript{2+} channels are fast-inactivating and function in triggering classical neurotransmitter release (Dunlap et al. 1995). The R-type Ca\textsuperscript{2+} current is resistant to known blockers of the previously mentioned Ca\textsuperscript{2+} channel types and may also have a role in transmitter release (Wu et al. 1998).

Due to an ~ 10,000-fold difference in Ca\textsuperscript{2+} concentration across the membrane of neurons, the opening of voltage-gated Ca\textsuperscript{2+} channels during action potential firing results in prominent Ca\textsuperscript{2+} influx (Levitan & Kaczmarek, 2002; Hille 2003). At rest, intracellular Ca\textsuperscript{2+} levels range between 50-300 nM, depending on the cell type, while stimulation causes these levels to rise to the low micromolar range (Wayman et al. 2008). At the ribbon synapse of retinal bipolar cells, the ready-releasable pool of vesicles requires Ca\textsuperscript{2+} microdomains to reach a minimum of 1 μM Ca\textsuperscript{2+} for release (Beaumont et al. 2005), whereas the squid giant synapse, action potential firing causes near-membrane Ca\textsuperscript{2+} microdomains to reach concentrations as high as 200-300 μM.
during transmitter secretion (Llinas et al. 1992).

An elevation in intracellular Ca\(^{2+}\), predominantly due to Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels, can be amplified through a phenomenon known as Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) (Endo et al. 1970; Fabiato & Fabiato, 1975; Gorman & Thomas, 1980; Smith et al. 1983; Neering & McBurney, 1984; Lipscombe et al. 1988). The Ca\(^{2+}\)-dependent opening of ryanodine receptors at the membrane of the endoplasmic reticulum enables the release of Ca\(^{2+}\) from this store since the Ca\(^{2+}\) concentration within the endoplasmic reticulum is ~1000-fold greater than the cytosol (Bardo et al. 2006). CICR can be diminished by blocking the receptors with ryanodine (Weber, 1968; Imagawa et al. 1987), and depleting Ca\(^{2+}\) with either an inhibitor of the endoplasmic reticulum Ca\(^{2+}\)-ATPase, cyclopiazonic acid (CPA) (Seidler et al. 1989), or the ryanodine receptor agonist, caffeine (Weber 1968; Rousseau et al. 1988). Although not traditionally considered as having a role in CICR, the mitochondria have recently been shown to be a key component. The very negative membrane potential of the mitochondria creates a strong driving force for Ca\(^{2+}\) to enter the organelle via the mitochondrial Ca\(^{2+}\) uniporter (MCU) (Johnson et al. 1981; Ylitalo et al. 2000; Kirichok et al. 2004). The mitochondria, in turn, release Ca\(^{2+}\) via Na\(^+\)/Ca\(^{2+}\) and H\(^+\)/Ca\(^{2+}\) exchangers (Pauceka & Jaburek, 2004). In the bag cell neurons of Aplysia, collapsing the mitochondrial membrane potential with carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) depletes mitochondrial Ca\(^{2+}\) and completely inhibits CICR (Geiger & Magoski, 2008; see section on ‘The bag cell neuron afterdischarge’).

The binding of a hormone to a metabotropic receptor stimulates dissociation of coupled G-proteins, initiating a cascade of events that eventually results in elevation of intracellular Ca\(^{2+}\) (Simon et al. 1991; Levitan & Kaczmarek, 2002). Activation of the membrane-bound enzyme, phospholipase C, requires interaction with the G-protein alpha subunit and leads to cleavage of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP\(_3\)) and diacylglycerol (DAG). IP\(_3\) diffuses throughout the cytosol where it binds to IP\(_3\) receptors on the endoplasmic reticulum membrane and triggers the efflux of stored Ca\(^{2+}\). DAG remains in the membrane, and in
concert with Ca\(^{2+}\) and phosphatidylinerine, activates protein kinase C (PKC), which alters a variety of target proteins by phosphorylation.

Many physiological responses to elevated intracellular Ca\(^{2+}\) are mediated by the Ca\(^{2+}\)-sensing transduction molecule, calmodulin (CaM). This ubiquitously-expressed, 17 kD protein binds Ca\(^{2+}\) at a pair of Ca\(^{2+}\)-binding motifs on each end, leading to a conformational change that promotes the interaction of Ca\(^{2+}\)/CaM with target proteins (Chin & Means, 2000). Ca\(^{2+}\)/CaM regulates numerous processes within the cell, including signalling pathways, structural proteins, ion channels, pumps, transcription factors and enzymes (Wayman et al. 2008). A family of kinases, known as CaM kinases, are activated by the binding of Ca\(^{2+}\)/CaM. Found predominantly in the brain, CaM kinases modify the function of many target proteins by the phosphorylation of Ser/Thr residues (Wayman et al. 2008). Figure 1 summarizes the main routes of Ca\(^{2+}\) entry into the cytosol and the subsequent consequences for the neuron.

**Ca\(^{2+}\)-dependent channel gating**

Changes in intracellular Ca\(^{2+}\) can be converted into changes in excitability via the activation of Ca\(^{2+}\)-dependent ion channels. The first description of a current dependent on increases in cytosolic Ca\(^{2+}\) was the Ca\(^{2+}\)-activated K\(^{+}\) current, discovered in *Aplysia* neurons (Meech & Strumwasser, 1970) and later found to be widespread (Sah & Faber, 2002). Ca\(^{2+}\)-activated K\(^{+}\) currents produce significant changes in the excitability of a neuron, notably by accelerating repolarization during an action potential and reducing the probability of bursting (Sah & Faber, 2002). These channels also provide an effective negative feedback mechanism for regulating Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels, which in turn can modulate neurotransmitter release at presynaptic terminals (Levitan, 1999). There are two types of identified Ca\(^{2+}\)-activated K\(^{+}\) channels: the BK and SK channel. BK channels are characterized by large single-channel conductances of 200 to 400 pS and a dependence on both Ca\(^{2+}\) and membrane potential for activation (Marty, 1981; McManus, 1991), while SK channels have smaller single channel
Figure 1. **Neuronal Ca\(^{2+}\) dynamics**

A schematic showing the regulation of intracellular Ca\(^{2+}\). Ca\(^{2+}\) enters through ligand- or voltage-gated Ca\(^{2+}\) channels. This prominent elevation in Ca\(^{2+}\) stimulates ryanodine receptors (RyR), which causes the endoplasmic reticulum to release Ca\(^{2+}\) that had previously been accumulated by the smooth endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA). Ca\(^{2+}\) is also taken up by the mitochondria via the mitochondrial Ca\(^{2+}\) uniporter (MCU) and released by Ca\(^{2+}\) exchangers (CaX\(\Delta\)). Additionally, Ca\(^{2+}\) can pass through the mitochondrial permeability transition pore (MPTP). Changes in Ca\(^{2+}\) fundamentally affect secretion, biophysics, and gene expression. Traditionally, mitochondrial Ca\(^{2+}\) is not thought to participate in secretion or biophysics. However, the endoplasmic reticulum has typically been implicated in these processes.
conductances of 2-20 pS and are insensitive to membrane potential (Romey & Lazdunski, 1984; Blatz & Magleby, 1986; Lang & Ritchie, 1987). Activation of BK channels requires the direct binding of Ca$^{2+}$ to the channel, presumably at a site within a series of negatively charged amino acids, known as the “Ca$^{2+}$ bowl” (Wei et al. 1994; Schreiber & Salkoff, 1997). Unlike BK channels, SK channels are indirectly activated by Ca$^{2+}$, with covalently-linked CaM as the mediator (Xia et al. 1998; Keen et al. 1999; Schumacher et al. 2001).

Cation channels, which are permeable to a combination of Na$^{+}$, K$^{+}$ and sometimes Ca$^{2+}$, provide depolarizing current that promotes bursting or contributes to a plateau potential (Kramer & Zucker, 1985; Swandulla & Lux, 1985; Partridge & Swandulla, 1988; Partridge et al. 1994; Morisset & Nagy, 1999). It is common for cation channels to be gated by intracellular Ca$^{2+}$, either directly or with CaM as an intermediate (Levitan, 1999). Activation of these channels often occurs at Ca$^{2+}$ levels of ~1 μM, yet they can remain responsive over a wide range of concentrations, nanomolar to micromolar (Yellen, 1982; Partridge & Swandulla, 1987, 1988; Razani-Boroujerdi & Partridge, 1993). An additional mode of cation channel regulation is through phosphorylation, as demonstrated by the PKC-induced increase in Ca$^{2+}$-sensitivity of the transient receptor potential/melastatin (TRPM4) cation channel (Launay et al. 2002; Nilius et al. 2005).

Transient receptor potential (TRP) channels belong to a superfamily that is composed of more than twenty cation channels having significant overlap in their genetic sequence (Montell, 2001). Found in many species and widely-expressed throughout the nervous system, TRP channels have been shown to be important in a diversity of physiological processes. Although sequence homology is shared among TRP channels, they vary greatly in their activating mechanisms, with Ca$^{2+}$/CaM having a role in activating some TRP channels.

Ca$^{2+}$ is even capable of providing negative feedback to limit the entry of Ca$^{2+}$ itself. As first discovered in Aplysia, incoming Ca$^{2+}$ initiates a process that leads to the inactivation of Ca$^{2+}$ channels (Tillotson, 1979), which involves the binding of Ca$^{2+}$ to CaM (Zühlke et al. 1999). Binding motifs for both Ca$^{2+}$ and CaM have been identified in the carboxy-terminal domain of the
α1C subunit of L-type Ca\(^{2+}\) channels that are essential for Ca\(^{2+}\)-dependent inactivation (Zühlke & Reuter, 1998). CaM appears to be fixed to the subunit, independent of Ca\(^{2+}\), while channel inactivation is dependent on Ca\(^{2+}\) binding to the fixed CaM and eliciting its interaction with the CaM-binding motif (Qin et al. 1999; Peterson et al. 1999). Ca\(^{2+}\)-dependent inactivation of Ca\(^{2+}\) channels is especially important where Ca\(^{2+}\) influx must be tightly regulated, such as in the heart or in synaptic transmission. Intracellular Ca\(^{2+}\) rises in response to hypoxic and ischemic insult to the myocardium (Steenbergen et al. 1987), which triggers inactivation of L-type Ca\(^{2+}\) channels (Yue et al. 1990). By preventing dramatic changes in cytosolic Ca\(^{2+}\), this negative feedback mechanism may hold off improper signalling during injury. Additionally, Ca\(^{2+}\) influx must be tightly controlled at the presynaptic terminal, as the release of neurotransmitters and neuropeptides is closely linked to Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels (Hille, 2001).

**Ca\(^{2+}\)-dependent neuroendocrine/peptide secretion**

From recordings of postsynaptic responses elicited by acetylcholine at the frog neuromuscular junction, del Castillo and Katz (1954) recognized that the postsynaptic response was due to presynaptic release of many discrete units, or ‘quanta,’ each containing equal amounts of neurotransmitter. By freezing the nerve terminal during the process of exocytosis, Heuser and colleagues (1979) were able to determine that each quantum corresponded to the exocytosis of a single vesicle. Only a portion of the entire pool of vesicles in the presynaptic terminal is ready for release upon stimulation, however, contrary to common assumption, these vesicles are not gathered close to the presynaptic membrane, but are located randomly throughout the vesicle pool (Rizzoli & Betz, 2004).

As proposed by the Ca\(^{2+}\) hypothesis of transmitter release, Ca\(^{2+}\) provides the critical link between electrical activity and the initiation of neurosecretion (Katz & Miledi, 1967a). Furthermore, the rate of transmitter release is directly proportional to the magnitude and duration of the voltage-dependent Ca\(^{2+}\) current (Llinas et al. 1981). Distinguishing between the influence of Ca\(^{2+}\) influx and membrane potential on secretion confirms that depolarization-induced Ca\(^{2+}\)
influx causes transmitter release, as opposed to the depolarization itself (Zucker & Haydon, 1988). Ca\(^{2+}\) also speeds up the priming of vesicles for release, and encourages pairing with Ca\(^{2+}\) channels, enabling them to respond rapidly to Ca\(^{2+}\) transients elicited by action potentials (Neher & Sakaba, 2008).

Formation of the SNARE (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors) complex is necessary for secretion and involves the tight coiling of specific proteins attached to both the vesicle and plasma membrane to draw these membranes together for release (Rettig & Neher, 2002; Smith et al. 2008). Three proteins are coiled within the complex: the plasma membrane protein, syntaxin, the vesicle-associated protein, synaptobrevin, and SNAP 25 (soluble NSF attachment protein 25 kD), which is bound to both syntaxin and Ca\(^{2+}\) channels to bring the vesicle closer to the channel (Jessell & Kandel, 1993; Rettig & Neher, 2002; Smith et al. 2008). During Ca\(^{2+}\) influx, the binding of Ca\(^{2+}\) to another vesicle-associated protein, synaptotagmin, triggers exocytosis (Jessel & Kandel, 1993; Smith et al. 2008). The SNARE complex is extremely stable in a low energy state and requires the ATPase, NSF, to unravel the complex following fusion (Jessell & Kandel, 1993; Rettig & Neher, 2002; Smith et al. 2008).

Like fast neurotransmitter release, neuroendocrine peptide release is dependent on Ca\(^{2+}\) influx and fails to occur when external Ca\(^{2+}\) is removed or voltage-gated Ca\(^{2+}\) channels are blocked (Hsu & Jackson, 1996; Whim et al. 1997; Branchaw et al. 1998; Sedej et al. 2004; Soldo et al. 2004). However, there is a notable delay between the initiation of Ca\(^{2+}\) influx and peptide release, suggesting that Ca\(^{2+}\) cannot induce release prior to attaining a certain threshold (Soldo et al. 2004). Prolonged bursts of action potentials are particularly efficient at triggering neuropeptide release, presumably because they generate the robust elevations in intracellular Ca\(^{2+}\) required to overcome the Ca\(^{2+}\) threshold (Bicknell & Leng, 1981; Hartzell, 1981; Soldo et al. 2004).

**Ca\(^{2+}\)-dependent gene transcription**

In addition to secretion, Ca\(^{2+}\) influx initiates a cascade of events that translates membrane activity into gene transcription (Greer & Greenberg, 2008). The importance of membrane activity
in the regulation of gene expression was first recognized in a study demonstrating that activation of the c-fos gene was dependent on the influx of Ca\^{2+} through L-type voltage-gated Ca\^{2+} channels in neuronal cell lines (Greenberg et al. 1986). Often implicated in Ca\^{2+}-dependent gene transcription, L-type Ca\^{2+} channels are well-suited to transmit Ca\^{2+} signals that reach the nucleus, due to their slow inactivation rate, high single-channel conductance, and localization at the soma (Westenbroek et al. 1990; Gallin & Greenberg, 1995; Catterall, 2000; Greer & Greenberg, 2008). Additionally, L-type Ca\^{2+} channels are closely associated with signalling proteins, such as CaM and calcineurin, both having roles in Ca\^{2+}-dependent gene transcription (Dolmetsch et al. 2001; Oliveria et al. 2007). Opening of these channels promotes Ca\^{2+} binding with CaM, which subsequently activates a variety of Ca\^{2+}/CaM-dependent proteins, including CaM kinase II (CaMKII) and calcineurin (Chin & Means, 2000). Once activated, CaMKII turns on cyclic adenosine monophosphate (cAMP)-responsive element binding proteins (CREB) via phosphorylation, while dephosphorylation by the phosphatase, calcineurin, triggers myocyte enhancer factor-2 (MEF2) (Sheng et al. 1991; Flavell et al. 2006; Shalizi et al. 2006). Prior to Ca\^{2+} influx, promoter-bound CREB and MEF2 attract complexes that repress transcription. However, in response to Ca\^{2+} influx, CREB and MEF2 are modified by CaM and calcineurin, respectively, which alleviates their repression of transcription (McKinsey et al. 2002; Chawla et al. 2003).

The first element found to regulate Ca\^{2+}-dependent transcription, known as the Ca\^{2+} response element (CaRE), is located within the c-fos promoter and is closely related to the cAMP response element (CRE) (Sheng et al. 1988). CREB generates transcription in response to elevated cAMP levels by binding to CRE; however, CREB also mediates Ca\^{2+}-dependent transcription by binding to CaRE (Sheng et al. 1990). Ca\^{2+}-dependent transcription of the early immediate genes, fos and jun, results in the expression of c-fos and c-jun proteins that together form the transcription-regulating AP-1 complex (Chiu et al. 1988; Halazonetis et al. 1988). Furthermore, Ca\^{2+} influx controls the rhythmic expression of the clock gene, Per1, in rat.
suprachiasmatic nucleus neurons (Lundkvist et al. 2005), as well as triggers CREB-phosphorylation, mediated by rapid translocation of CaM from the membrane to the nucleus, in hippocampal pyramidal neurons (Deisseroth et al. 1998; Mermelstein et al. 2001).

The bag cell neuron afterdischarge

The bag cell neurons of the marine mollusc, *Aplysia californica*, are neuroendocrine cells that control reproduction (Fig. 2). Providing a clear link between changes in neuronal activity and a distinct pattern of behaviours critical to the survival of the animal, they are well-suited for studying the neural control of fundamental behaviour. Additionally, the bag cell neurons offer a striking example of activity-dependent change in excitability and output. Located in two clusters of 200-400 neurons each, at the junction of the abdominal ganglion and the pleuroabdominal connectives, they are easily identifiable and conducive to electrophysiology based on their size (diameter 20-60 μm) (Kupfermann et al. 1966; Kupfermann 1967; Kupfermann & Kandel, 1970; Pinsker & Dudek, 1977; Dudek et al. 1979). Brief stimulation of the bag cell neurons initiates a prolonged burst of action potentials, referred to as the afterdischarge (Kupfermann 1967; Conn & Kaczmarek, 1989). A distinct pattern of firing occurs: a fast phase characterized by rapid firing (~5 Hz) lasting close to 1 min, and a slow phase with a lower firing frequency (~1 Hz) sustained over ~30 min (Kaczmarek et al. 1982; Fisher et al. 1994; Magoski & Kaczmarek, 2005). Subsequent to the afterdischarge, neurons undergo an approximately 18 h refractory period, during which another afterdischarge cannot be evoked (Kupfermann & Kandel, 1970; Kaczmarek et al. 1982). It appears that Ca\(^{2+}\) influx sets off the events that induce refractoriness, as neurons fail to become refractory following an afterdischarge in Ca\(^{2+}\)-free medium (Kaczmarek et al. 1982). In fact, Ca\(^{2+}\) influx through specific channels may be responsible for initiating the refractory period (Magoski et al. 2000).

The afterdischarge culminates in the release of several neuropeptides, including acidic peptide, an assortment of bag cell peptides, and egg-laying hormone (ELH) (Arch 1972a,b; Chiu et al. 1979; Scheller 1983; Loechner et al. 1990; Michel & Wayne, 2002; Jo et al. 2007). The
Figure 2. **Bag cell neurons control egg-laying behaviour in* Aplysia californica**

A, Egg-laying behaviour in *Aplysia californica*. A stringy egg-mass emerges near the head of the animal in the middle panel. The animal depicted here is typically ~ 15 cm in length and ~ 5 cm in width.

B, A dorsal view of *Aplysia* showing the location of major organs and the central nervous system.

C, A dorsal view of the abdominal ganglion highlighting the left and right bag cell neuron clusters. Stimulation of synaptic afferents in the pleural connectives triggers the bag cell neurons to afterdischarge and secrete peptides into circulation. The entire width of the ganglion represented here is typically 3-5 mm. All panels are modified from Kandel (1976).
latter is of particular importance because it triggers reproductive behaviours, primarily by acting at the ovotestis (Dudek & Tobe, 1978; Stuart et al. 1980). Secretion of ELH has been detected in response to an afterdischarge or a high $K^+$ stimulus from bag cell neurons in isolated abdominal ganglion, isolated bag cell clusters, and even from a few or single cultured bag cell neurons (Arch 1972a,b; Loechner et al. 1990; Jo et al. 2007). However, a depolarizing stimulus fails to evoke peptide release when Ca$^{2+}$ is removed from the external medium (Arch, 1972a,b). ELH is synthesized both in the soma and neurites in vivo and its production is increased following activity (Berry & Arch, 1981; Lee & Wayne, 2004). Although ELH and the bag cell neuron peptides are derived from the same prohormone, they are subsequently packaged into separate vesicles and sent to anatomically different neuronal processes (Sossin et al. 1990). The ELH expressed by the bag cell neurons in vivo is preserved in culture, which is the preparation used in the present study (Chiu & Strumwasser, 1981; White & Kaczmarek, 1997).

In concert with the other bag cell neuron peptides, ELH acts on central and peripheral targets to focus the behavioural program of the animal on reproduction by increasing respiratory pumping, redistributing blood flow, limiting locomotion, and suppressing defensive responses (Mayeri et al. 1979a,b; Stuart & Strumwasser, 1980; Mackey & Carew, 1983; Rothman et al. 1983; Ligman & Brownell, 1985; Schaefer & Brownell, 1986; Goldsmith & Byrne, 1993). Furthermore, the association between the afterdischarge and egg-laying is nearly incontrovertible, based on studies demonstrating that injection of either the homogenate of a bag cell neuron cluster, releasate from bag cell neurons, or ELH into an *Aplysia* causes egg-laying, egg-laying is always proceeded by an afterdischarge, stimulation of an afterdischarge in vivo causes egg-laying, and the removal of the bag cell neurons disrupts egg-laying (Kupfermann 1970; Pinsker & Dudek, 1977; Chiu et al. 1979; Stuart et al. 1980).

The afterdischarge is triggered by unknown chemical input and the passive spread of electrical charge throughout both bag cell neuron clusters, attributable to the direct electric coupling of bag cell neurons within a cluster and with cells of the opposite cluster (Blankenship &
Haskins, 1979; Brown et al. 1989). Accompanying the afterdischarge is the elevation of an array of second messengers, including Ca^{2+} (Woolum & Strumwasser, 1988; Fisher et al. 1994), cAMP (Kaczmarek et al. 1978), IP_3 (Fink et al. 1988), PKC (Wayne et al. 1999) and CaM kinase (DeRiemer et al. 1984). The activation of at least two cation channels provides the depolarizing drive for the afterdischarge: a Ca^{2+}-activated, voltage-independent cation channel that reverses near -40 and is triggered by Ca^{2+} influx via CaM kinase (Whim & Kaczmarek, 1998; Hung & Magoski, 2007) and a Ca^{2+}-activated, voltage-gated, Ca^{2+}-permeable cation channel that reverses near +50 and requires both PKC upregulation and src family tyrosine kinase down regulation for opening (Wilson et al. 1996, 1998; Magoski et al. 2002; Magoski & Kaczmarek, 2005; Lupinsky & Magoski, 2006; Gardam & Magoski, 2009). The afterdischarge is also associated with an increase in excitability and action potential broadening, partially due to inhibition of the delayed K^+ current, A-current, and Ca^{2+}-activated K^+ current via phosphorylation by cAMP-dependent protein kinase A (PKA) (Kaczmarek & Strumwasser, 1984; Strong 1984; Strong & Kaczmarek, 1986; Loechner & Kaczmarek, 1990; Zhang et al. 2004). Meanwhile, PKC mediates an increase in Ca^{2+} current during the afterdischarge to effectively increase action potential height and Ca^{2+} influx (DeRiemer et al. 1985; Strong et al. 1987; Knox et al. 1992; Geiger & Magoski 2008).

Intracellular Ca^{2+} rises during the fast phase of the afterdischarge, largely as a result of rapid Ca^{2+} influx through voltage-gated Ca^{2+} channels, and then plateaus during the slow phase, due in part to CICR (Fisher et al. 1994). IP_3 is present in elevated amounts during the afterdischarge and releases Ca^{2+} from the endoplasmic reticulum when injected into bag cell neurons, an effect that is prevented when heparin is used to block IP_3 receptors (Fink et al. 1988; Jonas et al. 1997). In culture, a short train of action potentials, designed to mimic the stimulus that triggers the afterdischarge, causes a rapid and transient rise in Ca^{2+}, but fails to elicit CICR (Geiger & Magoski, 2008) (Fig. 3). However, a longer train of action potentials, designed to mimic the fast phase of the afterdischarge, causes a rapid, large, transient rise in Ca^{2+} followed by CICR (Geiger & Magoski, 2008). Furthermore, CICR is completely blocked by mitochondrial
Figure 3. Ca\(^{2+}\) elevation induced by the fast phase of the afterdischarge

A, An extracellular recording of bag cell neurons in an intact cluster shows a 5 Hz, 10 s stimulus (at bar) elicits an afterdischarge. The afterdischarge lasts for approximately 30 minutes and consists of both a fast and slow phase of action potential firing. The length of the recording was truncated for display purposes. Courtesy of Dr. Neil Magoski.

B, Upper, Ratiometric fura PE3 imaging of the intracellular Ca\(^{2+}\) response to a 5 Hz, 1 min train of action potentials: a rapid, transient Ca\(^{2+}\) elevation followed by a prolonged plateau consistent with Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). Lower, Simultaneous recording of membrane potential during the 5 Hz, 1 min train. Modified from Geiger & Magoski (2008).
depolarization with FCCP or inhibition of mitochondrial Ca²⁺ exchange prior to the delivery of the train, suggesting that the mitochondria plays a critical role in CICR, presumably by sequestering the Ca²⁺ that enters during the train and releasing it through exchange (Geiger & Magoski, 2008). This Ca²⁺ that is released by the mitochondria may in turn activate ryanodine receptors on the endoplasmic reticulum, since CICR can be partially blocked by inhibiting ryanodine receptors with dantrolene or CPA-induced depletion of endoplasmic reticulum Ca²⁺ (Geiger & Magoski, 2008). But what is the consequence of Ca²⁺ influx and release for bag cell neuron function?

Hypothesis and significance

The aim of this thesis is to examine the consequences of electrically- or pharmacologically-induced Ca²⁺ changes on secretion and excitability in cultured bag cell neurons. Using whole-cell voltage-clamp, whole-cell capacitance tracking, sharp-electrode current-clamp, and ratiometric intracellular Ca²⁺ imaging, I will test the following hypotheses:

1) Activity-dependent elevation of Ca²⁺ elicits secretion.

2) Ca²⁺ liberated from intracellular stores activates a membrane current.

Delivery of a train of action potentials mimicking the fast phase of the afterdischarge evokes an elevation in intracellular Ca²⁺ with an early, transient phase, dominated by Ca²⁺ influx through voltage-gated Ca²⁺ channels, and a subsequent plateau phase, which is due to release from intracellular stores (Geiger & Magoski, 2008). If the elevation in Ca²⁺ during the fast phase is capable of initiating secretion, this would provide a link between the activity of membrane Ca²⁺ channels and the control of a fundamental behaviour of the animal. The prolonged plateau of elevated intracellular Ca²⁺, following the transient peak, is a result of mitochondria releasing Ca²⁺ that had been sequestered during the fast phase, as well as Ca²⁺-induced Ca²⁺ release from the endoplasmic reticulum (Geiger & Magoski, 2008). If the Ca²⁺ released by intracellular stores
during the slow phase activates membrane currents, these currents may provide the depolarizing drive to the afterdischarge itself.
Chapter 2: Materials and Methods

Animals and cell culture

Primary cultures of isolated bag cell neurons were obtained from adult *Aplysia californica*, weighing 150-500 g. Animals were purchased from Marinus (Long Beach, CA, USA) or Santa Barbara Marine Biologicals (Santa Barbara, CA, USA) and housed in an ~ 300 l aquarium containing continuously circulating, aerated artificial seawater (Instant Ocean; Aquarium Systems; Mentor, OH, USA) at 14-16° on 12/12 h light/dark cycle and fed Romaine lettuce 5x/week. Following anaesthesia by injection of isotonic MgCl₂ (~50% of body weight), the abdominal ganglion was removed and treated for 18 h with neutral protease (13.33 mg/ml; 165859; Roche Diagnostics, Indianapolis, IN, USA) dissolved in tissue culture artificial seawater (tcASW; containing in mM: 460 NaCl, 10.4 KCl, 11 CaCl₂, 55 MgCl₂, 10 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), glucose (1 mg/ml), penicillin (100 U/ml), and streptomycin (0.1 mg/ml), pH 7.8 with NaOH). The ganglion was then transferred to fresh tcASW and the two bag cell neuron clusters were dissected from their surrounding connective tissue. Using a fire-polished Pasteur pipette and gentle trituration, neurons were dispersed in tcASW onto 35 x 10 mm polystyrene tissue culture dishes (430165; Corning, Corning, NY, USA). Cultures were maintained in tcASW or simple culture medium (SCM; composition as per tcASW plus minimum essential medium (MEM) vitamins (0.5X; 11120052; Gibco/Invitrogen; Grand Island, NY, USA), MEM non-essential amino acids (0.2X; 11400050; Gibco/Invitrogen), and MEM essential amino acids without L-glutamine (0.2X; 1130051; Gibco/Invitrogen) for 1-3 d in a 14°C incubator. Experiments were carried out at 22°C. Salts were obtained from Fisher Scientific (Ottawa, ON, Canada), ICN (Irvine, CA, USA), or Sigma-Aldrich (St. Louis, MO, USA).

Whole-cell voltage-clamp recordings

Voltage-clamp recordings were made using an EPC-8 amplifier (HEKA Electronics; Mahone Bay, NS, Canada) and the tight-seal, whole-cell method. Microelectrodes were pulled
from 1.5 mm internal diameter, borosilicate glass capillaries (TW150F-4; World Precision Instruments, Sarasota, FL, USA) and had a resistance of 1-3 MΩ when filled with various intracellular salines. Pipette junction potentials were nulled immediately before seal formation. Pipette and neuronal capacitive currents were cancelled, and following break through, the series resistance (3-5 MΩ) was compensated to 70-80% and monitored throughout the experiment. Cell capacitance was usually derived from the EPC-8 whole-cell capacitance compensation (but see Capacitance Tracking). Current was filtered at 1 KHz by the EPC-8 built-in Bessel filter and sampled at 2 KHz using an IBM-compatible personal computer, a Digidata 1300 analogue-to-digital converter (Axon Instruments/Molecular Devices; Sunnyvale, CA, USA) and the Clampex acquisition program of pCLAMP 8.1 (Axon Instruments). Clampex was also used to maintain and change the membrane potential. Most recordings were done in normal ASW (nASW; composition as per tcASW, but with glucose and antibiotics omitted) with microelectrodes filled with regular intracellular saline (composition in mM: 500 K-aspartate, 70 KCl, 1.25 MgCl₂, 10 HEPES, 11 glucose, 10 glutathione, 5 ethylene glycol bis(aminoethyl ether) tetraacetic acid (EGTA), 5 ATP (grade 2, disodium salt; Sigma), and 0.1 GTP (type 3, disodium salt; Sigma); pH 7.3 with KOH; free Ca²⁺ concentration set to 300 nM). In some experiments, the intracellular Ca²⁺ was buffered using a high-EGTA intracellular saline with 20 mM EGTA, 5 mM MgCl₂, and a free Ca²⁺ concentration set at 35 nM. Ca²⁺ concentrations were calculated using WebMaxC (http://www.stanford.edu/~cpatton/webmaxcS.htm). Both of the above intracellular salines had a calculated junction potential of 15 mV versus nASW, which was compensated by off-line subtraction.

**Sharp-electrode current-clamp recording**

Current-clamp recordings were made using an Axoclamp 2B amplifier (Axon Instruments/Molecular Devices) and the sharp-electrode, bridge-balanced method. Microelectrodes were pulled from 1.2 mm internal diameter, borosilicate glass capillaries (IB120F-4; World Precision Instruments) and had a resistance of 7-12 MΩ when filled with 2 M
K-acetate (supplemented with 100 mM KCl and 10 mM HEPES; pH=7.3 with KOH). Voltage signals were filtered at 3 kHz and sampled at 2 kHz. Current was delivered either from the amplifier, Clampex, or a S88 stimulator (Grass; Warwick, MA, USA).

**Ca²⁺ imaging**

The Ca²⁺-sensitive dye, fura-PE3 (K⁺ salt; 0110; Teflabs, Austin, TX, USA; Vorndran *et al.* 1995), was injected via sharp-electrode using a PMI-100 pressure microinjector (Dagan; Minneapolis, MN, USA), while simultaneously monitoring membrane potential with the Axoclamp. Microelectrodes (as per Sharp-electrode current-clamp) had a resistance of 15-30 MΩ when the tip was filled with 10 mM fura-PE3 then backfilled with 3 M KCl. Injections required 3-10, 200-300 ms pulses at 50-100 kPa to fill the neurons with an optimal amount of dye - estimated to be 50-100 µM. All neurons used for imaging showed resting potentials of -50 to -60 mV and displayed action potentials that overshot 0 mV following depolarizing current injection (0.5-1 nA, directly from the amplifier). After dye injection, neurons were allowed to equilibrate for at least 1 h. Imaging was performed using a Nikon TS100-F inverted microscope (Nikon; Mississauga, ON, Canada) equipped with Nikon Plan Fluor 10X (numerical aperture (NA) = 0.5), 20X (NA=0.5) or 40X (NA=0.6) objectives. The light source was a 75 W Xenon arc lamp and a multi-wavelength DeltaRAM V monochromatic illuminator (Photon Technology International; London, ON, Canada) coupled to the microscope with a UV-grade liquid-light guide. Excitation wavelengths were 340 and 380 nm. Between acquisition episodes, the excitation illumination was blocked by a shutter, which along with the excitation wavelength, was controlled by an IBM-compatible personal computer, a Photon Technology International computer interface, and ImageMaster Pro software (version 1.49; Photon Technology International). The emitted light passed through a 510/40 nm barrier filter prior to being detected by a Photon Technology International IC200 intensified charge coupled device camera. The camera intensifier voltage was set based on the initial fluorescence intensity of the cells at the beginning of each experiment and maintained constant thereafter. The camera black level was set prior to an experiment using the camera
controller such that at a gain of 1 and with no light going into the camera there was a 50:50
distribution of blue and black pixels on the image display. Fluorescence intensities were sampled
at 60 sec intervals using regions of interests (ROIs) defined over the neuronal somata prior to the
start of the experiment and averaged 8 frames per acquisition. The focal plane (Z-axis) was set to
approximately the middle of the neuron. The ratio of the emission following 340 and 380 nm
excitation (340/380) was taken to reflect free intracellular Ca\textsuperscript{2+}, and saved for subsequent
analysis. The black level determination, image acquisition, frame averaging, emitted light ROI
sampling, and ratio calculations were carried out using ImageMaster Pro.

**Capacitance tracking**

As an indicator of secretion, membrane capacitance was tracked under whole-cell
voltage-clamp using the time-domain method (Gillis, 1995). Whole-cell recording conditions
were the same as described previously, except that series resistance and whole-cell capacitance
were not compensated. The holding potential was -80 mV, from which 20 mV, 100 ms pulses to -
60 mV were delivered at 0.5 Hz. With pipette capacitance compensated, the membrane test
function of Clampex was employed to determine the initial current (I<sub>i</sub>) just after the start of the
step and the steady-state current (I<sub>ss</sub>) just prior to the end of the step, as well as fit the time
constant of current decay (τ) from initial to steady-state with a single-exponential. These three
values were then used to calculate access resistance, membrane resistance, and membrane
capacitance (see Analysis). To increase accuracy and improve the signal-to-noise ratio, current
traces were cumulatively averaged (10 pulses per average) before each calculation. The -80 mV
holding potential was chosen to avoid activation of any voltage-gated Ca<sup>2+</sup> channels during the 20
mV step (Tam & Magoski, 2007). Most of the capacitance tracking recordings were performed in
Ca<sup>2+</sup>-Cs<sup>+</sup>-TEA ASW, where NaCl and KCl were replaced by TEA-Cl and CsCl, respectively (pH
7.8 with CsOH) and using Cs<sup>+</sup>-aspartate intracellular saline where the K<sup>+</sup> was replaced by Cs<sup>+</sup> and
no Ca<sup>2+</sup> was added (pH 7.3 with CsOH). In some instances, intracellular saline contained a high
concentration (20 mM) of the Ca<sup>2+</sup> buffer, EGTA, as well as 5 mM MgCl<sub>2</sub>, and a free Ca<sup>2+</sup>
concentration of 35 nM. This intracellular saline had a calculated junction potential of 20 mV vs the Ca\(^{2+}\)-Cs\(^+\)-TEA ASW, which was compensated by offline subtraction.

**Reagents and drug application**

Solution exchanges were accomplished by manual perfusion using a calibrated transfer pipette to first exchange the bath (tissue culture dish) solution. Then, in most cases where a drug was applied, a small volume (<10 µl) of concentrated stock solution was mixed with a larger volume of saline (approximately 100 µl) that was initially removed from the bath, and this mixture was then pipetted back into the bath. Care was taken to add drugs near the side of the dish and as far away as possible from the neurons. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 21857; Sigma-Aldrich), antimycin A (A8674; Sigma-Aldrich), cyclopiazonic acid (CPA; C1530; Sigma-Aldrich or 239805; Calbiochem, San Diego, CA, USA), bafilomycin A (B1793, Sigma-Aldrich), oligomycin A (75351; Sigma-Aldrich), carbonyl cyanide 3-chlorophenylhydrazone (CCCP; C2759, Sigma-Aldrich), and cyclosporin A (239835; Calbiochem) all required dimethyl sulfoxide (DMSO; BP231; Fisher) as a vehicle. The maximal final concentration of DMSO was 0.01 µM, which in control experiments had no effect on current, voltage, or intracellular Ca\(^{2+}\). Agents made up in distilled water included NiCl\(_2\) (N6136; Sigma-Aldrich), GdCl\(_3\) (G7532; Sigma-Aldrich), and 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride (SKF-96365; 567310; Calbiochem). N-ethylmaleimide (NEM; E3876; Sigma-Aldrich) was dissolved as a stock in 100% ethanol.

**Analysis**

Clampfit, a program of pClamp, was used to measure current, voltage, or membrane capacitance. For most of these experiments, the analysis involved comparing the average value during a steady-state baseline of 1-5 min, with the average value from regions that had reached a peak for 5-30 s or remained stable for 5-10 min, after the delivery of a drug or stimulus. The current-voltage relationships, as well as reversal potentials, were determined from a difference current generated by a -120 to 0 mV ramp given before and after FCCP. To derive the difference
current, the ramp current recorded prior to the addition of FCCP was subtracted from the ramp current taken at the peak of the response induced by FCCP. All measurements of current were normalized to cell size (pA/pF) by dividing by whole-cell capacitance. Conductance was derived using Ohm’s law and the current during a 200 ms step from -60 mV to -70 mV. The % change was calculated from the conductance before and after addition of FCCP.

Membrane capacitance was calculated as per Gillis (1995) using the formula: $C_m = \tau(1/R_a + 1/R_m)$, where $\tau = \text{time constant of current decay during the voltage step from } -80 \text{ to } -60 \text{ mV}$, $R_a =$ access resistance, and $R_m =$ membrane resistance. $R_a$ was calculated using the formula: $R_a = V_s/I_i$, where $V_s =$ the magnitude of the voltage step (20 mV) and $I_i =$ the initial current flowing during the step. $R_m$ was calculated using the formula: $R_m = (V_s - R_a \cdot I_{ss})/I_{ss}$, where $I_{ss} =$ the steady-state current flowing during the step.

Origin (version 7; OriginLab Corporation; Northampton, MA, USA) was used to import and plot ImageMaster Pro files as line graphs. For intracellular Ca\(^{2+}\) experiments, analysis usually compared the steady-state value of the baseline 340/380 ratio with the ratio from regions that had reached a peak. Hill curve fits were also generated in Origin and provided the 50% effective concentration (the concentration that is required for 50% of maximal activation) as well as the Hill coefficient.

Summary data are presented as the mean ± standard error of the mean. Statistics were performed on raw data, normalized data, or percent change values using Instat (version 3.0; GraphPad Software, San Diego, CA, USA). The Kolmogorov-Smirnov method was used to test data sets for normality. If the data were normal, Student’s paired or unpaired t-test was used to test for differences between two means, while a standard one-way analysis of variance (ANOVA) with Bonferroni’s post-hoc test were used to test for differences between multiple means. In cases where the data were not normal, the Welch correction was applied to Student’s paired or unpaired t-test, when testing for differences between two means, while a nonparametric Kruskal-Wallis
ANOVA with Dunn’s multiple comparisons test was used to test for differences between multiple means. Data was considered significantly different if the p-value was <0.05.
Chapter 3: Results

Ca\textsuperscript{2+} entry causes an increase in bag cell neuron membrane capacitance

Excitation of the bag cell neurons elicits an approximate 30 min afterdischarge, which initiates reproduction through the secretion of egg-laying hormone (ELH). The afterdischarge has a fast phase and a slow phase. As described by Geiger and Magoski (2008), a fast phase-like stimulus evokes an immediate and prominent influx of Ca\textsuperscript{2+} through voltage-gated Ca\textsuperscript{2+} channels. To investigate if Ca\textsuperscript{2+} influx was capable of triggering secretion, capacitance tracking of cultured bag cell neurons was used as an assay and the fast phase mimicked with a 5 Hz, 1 min train of 75 ms pulses from -80 to -0 mV. It is well established that capacitance tracking indirectly measures secretion, based on the assumption that vesicle fusion causes a detectable change in membrane surface area (Neher & Marty, 1982; Tse et al. 1997; Neves et al. 2001; Klyachko & Jackson, 2002). In solutions that isolate Ca\textsuperscript{2+} currents (Ca\textsuperscript{2+}-Cs\textsuperscript{-}-TEA ASW external and Cs\textsuperscript{-}-aspartate internal) (Fig. 4A inset), the 5 Hz, 1 min train caused a robust increase in membrane capacitance (n=8) (Fig. 4A).

Ba\textsuperscript{2+} is commonly used as a substitute for Ca\textsuperscript{2+} because it readily passes through Ca\textsuperscript{2+} channels (Hagiwara et al. 1974; Hille, 2001); however, Ca\textsuperscript{2+} is often expressly required to initiate active processes, such as channel gating or secretion (Miledi, 1966; Shin et al. 2003; Lupinsky & Magoski, 2006). I replaced extracellular Ca\textsuperscript{2+} with Ba\textsuperscript{2+} (n=11) and found that it abolished the capacitance elevation induced by the train (Fig. 4A). The rise in membrane capacitance triggered by the train was significantly greater when Ca\textsuperscript{2+} was present in the external (Fig. 4B). These observations point to a fundamental role for Ca\textsuperscript{2+} influx through voltage-gated Ca\textsuperscript{2+} channels in eliciting apparent secretion from bag cell neurons.

Next, I attempted to determine if there was an optimal pulse duration for generating a capacitance change in response to the 5 Hz, 1 min train. The train was delivered to cultured bag cell neurons, voltage-clamped at -80 mV, with varying pulse durations (50, 75, 100, or 150 ms).
Figure 4. Ca\textsuperscript{2+} influx initiates an increase in membrane capacitance of bag cell neurons

**A, Upper,** Cultured bag cell neurons are whole-cell voltage-clamped at a holding potential (HP) of -80 mV using solutions to isolate Ca\textsuperscript{2+} currents. Membrane capacitance is tracked with the time domain technique. A 5 Hz, 1 min train of 75 ms pulses from -80 mV to 0 mV causes an elevation in membrane capacitance. **Lower,** This effect is eliminated when Ca\textsuperscript{2+} in the external solution is replaced with Ba\textsuperscript{2+}. The gap during the train is due to the tracking software being incompatible with the large change in membrane conductance produced by the depolarization. The inset displays an example of Ca\textsuperscript{2+} currents during a 5 Hz, 1 min train. All 300 traces are superimposed.

**B,** Summary data comparing the mean percent change in capacitance following the train shows a significant difference when external Ca\textsuperscript{2+} is substituted with Ba\textsuperscript{2+} (two-tailed unpaired t-test; Welch corrected). The percent change in capacitance reflects the peak change normalized to baseline. For this and subsequent bar graphs, data represent the mean ± standard error with the n value indicated in, above, or below the bars.

**C,** Summary data of the mean percent change in capacitance following a 5 Hz, 1 min train with a pulse duration of 50 ms, 75 ms, 100 ms, or 150 ms. Varying pulse duration does not significantly (ns) alter the change in capacitance produced by the train (one-way ANOVA, Bonferroni’s multiple comparisons test).
However, the amplitude of the change in membrane capacitance did not vary significantly with
pulse duration (Fig. 4C). For consistency, the pulse duration of 75 ms was chosen for all
subsequent experiments involving the 5 Hz, 1 min train. Moreover, Hung and Magoski (2007)
found that a 75 ms pulse duration was optimal for initiating activity-dependent changes to
excitability in bag cell neurons. In a few neurons, I also examined the effect of a 5 Hz, 10 s train,
but this shorter stimulus failed to alter membrane capacitance (data not shown; n=4).

**A general Ca\(^{2+}\) channel blocker eliminates the increase in capacitance**

The Ca\(^{2+}\) influx triggered by the 5 Hz, 1 min train appears to initiate secretion. To further
test whether Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels is responsible, I blocked the channels
before delivering the train. A 5 Hz, 1 min train was delivered twice, separated by approximately
15 min, to the same cultured bag cell neuron (n=10). The capacitance change produced by the
second stimulus was analysed relative to the capacitance change produced by the first stimulus.
The purpose of administering two trains was to first confirm that the individual neuron was
capable of responding with a change in membrane capacitance before attempting to block the
response with Ni\(^{2+}\). Each train evoked a rise in membrane capacitance, although the second
increase was consistently smaller than the first (Fig. 5A). Ni\(^{2+}\) has commonly been used to block
Ca\(^{2+}\) channels (Byerly *et al.* 1985; McFarlane & Gilly, 1998), and when delivered at a
concentration of 10 mM prior to the second train it completely abolished the subsequent change in
capacitance (n=5) (Fig. 5B). Previous work in our laboratory has shown 10 mM Ni\(^{2+}\) to be a
saturating concentration for Ca\(^{2+}\) channel block in bag cell neurons (Hung and Magoski 2007).
The consistent elimination of the capacitance increase induced by the train in the presence of Ni\(^{2+}\)
clearly reached significance (Fig. 5C). This was the case with either Ca\(^{2+}\)-Cs\(^{+}\)-TEA ASW or
simple culture medium in the bath (Fig. 5D). Subsequently, I did not test each individual neuron
for a capacitance change prior to an experiment because the responsiveness of a neuron could be
reliably predicted based on the animal. This is to say, I consistently observed that either all or
none of the bag cell neurons from a particular animal responded to the train with an increase in
Figure 5. Ni\(^{2+}\) blocks the increase in membrane capacitance

**A.** Two consecutive 5 Hz, 1 min trains delivered to a single neuron at -80 mV and bathed in Ca\(^{2+}\)-Cs\(^{-}\)-TEA ASW, each produce a change in capacitance. The second elevation is consistently smaller than the first. A time of 14 min separates delivery of the stimuli.

**B.** The addition of 10 mM Ni\(^{2+}\), a common Ca\(^{2+}\) channel blocker, prior to the second train eliminates the change in capacitance.

**C and D.** Summary data of the second response over the first (mean percent change) indicates that Ni\(^{2+}\) abolishes the capacitance change elicited by the train with either Ca\(^{2+}\)-Cs\(^{-}\)-TEA ASW (one-tailed unpaired t-test) or culture medium as the external (one-tailed unpaired t-test).
membrane capacitance.

**Buffering intracellular Ca\(^{2+}\) attenuates the increase in membrane capacitance**

There appears to be a strong link between Ca\(^{2+}\) entering the cell during the train and the subsequent elevation in membrane capacitance. To determine whether the train-induced increase in capacitance required an elevation of intracellular Ca\(^{2+}\), cultured bag cell neurons were dialysed for 10 min with intracellular solution containing either a regular (5 mM) or high (20 mM) concentration of the slow Ca\(^{2+}\) buffer, EGTA (Smith *et al.* 1984; Naraghi, 1997), prior to delivery of the train. When intracellular Ca\(^{2+}\) was buffered with high-EGTA (n=10), the increase in membrane capacitance evoked by the 5 Hz, 1 min train was attenuated by nearly half compared with the response using a regular concentration of EGTA (n=9) (Fig. 6A). This effect readily achieved significance (Fig. 6B) and is consistent with the change in membrane capacitance being dependent on elevated intracellular Ca\(^{2+}\).

**An alkylation agent inhibits the increase in membrane capacitance**

Blocking one of the steps leading to vesicle fusion with the membrane should attenuate the capacitance increase elicited by the train. N-ethylmaleimide (NEM) is an alkylation agent that disrupts protein sulfhydryl groups and has the capacity to block vesicle fusion with the membrane (Block *et al.* 1988; Han *et al.* 1999). Considering this, I pretreated cultured bag cell neurons for 30 min with either ethanol (the vehicle) or 100 μM NEM, and subsequently delivered a 5 Hz, 1 min train under whole-cell voltage-clamp at -80 mV. Following NEM treatment (n=5), the train produced an increase in capacitance that was diminished by two-thirds compared with ethanol-treated neurons (n=5) (Fig. 7A). The mean data showed that this reduction met the level of significance (Fig. 7B). Of course, I cannot assume that this is the only effect of NEM, as there are many proteins containing sulfhydryl groups that could be modified. In fact, NEM may indirectly modulate Ca\(^{2+}\) currents in *Aplysia* neurons, via alkylation of cysteine residues on the subunit of a G-protein (Fryer 1992). If NEM was also blocking Ca\(^{2+}\) influx, this could contribute to any observed reduction in the train-induced rise in capacitance. To examine this, I compared Ca\(^{2+}\)
Figure 6. **Buffering intracellular Ca\(^{2+}\) reduces the increase in membrane capacitance**

A, Whole-cell, voltage-clamp recordings at a holding potential of -80 mV show the delivery of a 5 Hz, 1 min train to separate neurons with either regular or high-EGTA internal in the pipette. In comparison with regular internal solution (5 mM EGTA; upper trace), the rise in membrane capacitance is markedly reduced when intracellular Ca\(^{2+}\) is buffered by high-EGTA (20 mM; lower trace). The train is delivered to each neuron following 10 min dialysis.

B, Summary data of the mean percent change in membrane capacitance shows that the increase induced by the train is lowered significantly when regular intracellular saline is substituted with high-EGTA saline (one-tailed unpaired t-test).
Figure 7. An alkylating agent inhibits the increase in membrane capacitance

A, Separate neurons are whole-cell, voltage-clamped at -80 mV and treated for 30 minutes with either ethanol (EtOH) or 100 μM N-ethylmaleimide (NEM), prior to the delivery of a 5 Hz, 1 min train. The elevation in membrane capacitance elicited by the train is diminished after treatment with NEM (lower trace) versus treatment with ethanol (upper trace).

B, Summary data shows a significant reduction in the mean percent increase in capacitance evoked by the train following NEM, as opposed to incubation in ethanol (two-tailed unpaired t-test).
currents during a 5 Hz, 1 min train after 30 min of pretreatment with either ethanol or 100 μM NEM. Although the average amplitude of total Ca$^{2+}$ current during a 5 Hz, 1 min train following treatment with NEM (n=5) was lower than with ethanol (n=5), this difference did not reach significance (ethanol = -658.0 ± 111.1 nA/nF vs NEM = -381.2 ± 85.8 nA/nF; p >0.05, two-tailed unpaired t-test).

FCCP, but not CPA, elevates membrane capacitance

Geiger and Magoski (2008) also described an elevation in intracellular Ca$^{2+}$ that lasted several minutes following the robust increase triggered by a 5 Hz, 1 min train of action potentials. This prolonged elevation was due to release of Ca$^{2+}$ from both the mitochondria and the endoplasmic reticulum. To examine the role of the mitochondria in secretion, I tracked capacitance in cultured bag cell neurons while using FCCP to deplete the organelle of Ca$^{2+}$. FCCP is a protonophore that collapses the mitochondrial membrane potential, which normally creates the driving force for Ca$^{2+}$ entry; in the absence of voltage, Ca$^{2+}$ leaks out (Heytler & Prichard, 1962; Simpson & Russell, 1996). In response to 20 μM FCCP (n=11), I observed an elevation in membrane capacitance that returned to baseline after a few minutes (Fig. 8A). I also investigated whether Ca$^{2+}$ from the endoplasmic reticulum was capable of stimulating secretion by depleting that store of Ca$^{2+}$ using the endoplasmic reticulum Ca$^{2+}$-ATPase blocker, CPA (Seidler et al. 1989). Unlike FCCP, 20 μM CPA (n=6) did not induce an increase in membrane capacitance (Fig. 8A). The difference between the effect of FCCP and CPA on membrane capacitance reached the level of significance (Fig. 8B).

It is possible that the Ca$^{2+}$ released into the cytosol from mitochondrial depletion by FCCP activates Ca$^{2+}$-dependent channels, of which there are several in bag cell neurons (Zhang et al. 2002; Lupinsky & Magoski, 2006; Gardam et al. 2008). As detailed in subsequent sections, FCCP does in fact open a potentially Ca$^{2+}$-permeable cation channel. This Ca$^{2+}$ entry could be responsible for the change in membrane capacitance, rather than mitochondrial Ca$^{2+}$ itself. To
Figure 8. **FCCP causes an increase in membrane capacitance**

**A**, Whole-cell, voltage-clamp recording at a holding potential of -60 mV in nASW indicates that depleting mitochondrial Ca\(^{2+}\) stores by collapsing organelle membrane potential with 20 μM FCCP transiently elevates membrane capacitance. In a separate neuron, depleting endoplasmic reticulum Ca\(^{2+}\) stores with 20 μM of the Ca\(^{2+}\)-ATPase blocker, CPA, does not change capacitance. The artifact at the beginning of each trace is due to drug addition.

**B**, Summary data comparing the mean percent change in membrane capacitance due to FCCP and CPA shows a significant difference (two-tailed unpaired t-test). To calculate percent change in capacitance, peak response was normalized to baseline.

**C**, Summary data shows that the mean percent increase in membrane capacitance induced by FCCP is not significantly different in Ca\(^{2+}\)-containing nASW versus an absence of external Ca\(^{2+}\) in cfASW (two-tailed unpaired t-test).
distinguish between Ca\(^{2+}\) influx and Ca\(^{2+}\) release, cultured bag cell neurons were bathed in either Ca\(^{2+}\)-containing nASW (n=7) or Ca\(^{2+}\)-free ASW (cfASW) (n=9), while administering 20 \(\mu\)M FCCP. There was no significant difference between the rise in membrane capacitance triggered by FCCP in nASW versus cfASW (Fig. 8C), implying that mitochondrial Ca\(^{2+}\) directly affects secretion.

**FCCP, but not CPA, depolarizes bag cell neurons**

As alluded to, I was also interested in the effects of released Ca\(^{2+}\) on other processes within the neuron. Geiger and Magoski (2008) made a preliminary report that the depletion of mitochondrial Ca\(^{2+}\) by FCCP depolarized bag cell neurons. To investigate this quantitatively, the membrane potential of cultured bag cell neurons was recorded using sharp-electrode current-clamp in nASW. As expected, 20 \(\mu\)M FCCP (n=18) triggered a pronounced depolarization of approximately 35 mV (Fig. 9A). On occasion, a short burst of action potentials was observed along with the depolarization. Additionally, I treated bag cell neurons with 20 \(\mu\)M CPA (n=4) to test whether depletion of Ca\(^{2+}\) from the endoplasmic reticulum had a similar effect on membrane potential. However, there was essentially no change in membrane potential in response to CPA (Fig. 9A). The depolarizing effect of FCCP was significant in comparison with CPA (Fig. 9B).

Many of the following experiments were performed using the whole-cell method, instead of sharp-electrode. When using whole-cell, an ATP-containing solution inside the pipette is dialysed with the contents of the cell, which is not the case for sharp-electrode. As whole-cell is potentially less damaging to the cell membrane, cell health could create variability. To control for this, I delivered 20 \(\mu\)M FCCP to cultured bag cell neurons in current-clamp, using the whole-cell configuration (n=8). There was no significant discrepancy in the average change in membrane potential produced by FCCP in whole-cell versus sharp-electrode (Fig. 9B).
Figure 9. **FCCP, but not CPA, depolarizes bag cell neurons**

**A**, Sharp-electrode, current-clamp recordings from two separate bag cell neurons show that depleting Ca^{2+} from the mitochondria with 20 μM FCCP, induces depolarization and spiking (left), but depleting endoplasmic reticulum Ca^{2+} stores with 20 μM CPA does not (right).

**B**, Summary data of the mean change in membrane potential shows that the effect of FCCP readily meets the level of significance when compared with CPA; however, the effect of FCCP is not significantly different when whole-cell (WC), rather than sharp-electrode (SE), is employed for the current-clamp recordings (Kruskal-Wallis nonparametric ANOVA; Dunn’s multiple comparisons test).
**FCCP, but not CPA, causes an inward current**

To understand these membrane potential effects, I examined whether depletion of Ca\(^{2+}\) from the mitochondria or the endoplasmic reticulum induced a current. Cultured bag cell neurons were whole-cell voltage-clamped at a holding potential of -60 mV with regular intracellular saline (K'-aspartate based; 300 nM free Ca\(^{2+}\)) in the pipette and nASW in the bath. The depletion of mitochondrial Ca\(^{2+}\) with 20 μM FCCP (n=10) consistently generated a slow, inward current of approximately 1 pA/pF (Fig. 10A). Under the same conditions, depletion of endoplasmic reticulum Ca\(^{2+}\) with 20 μM CPA (n=9) failed to evoke a current of significant amplitude in comparison with FCCP (Fig. 10A), nor did DMSO (n=7), the vehicle used for both drugs (Fig. 10B). Moreover, store-interaction or differential Ca\(^{2+}\) handling did not appear to be involved, as pretreatment with CPA (n=9) did not significantly alter the amplitude of the FCCP-induced current (Fig. 10B).

**The FCCP-induced current is concentration-dependent**

The 20 μM dose of FCCP represented a concentration our laboratory (Gardam et al. 2008; Geiger & Magoski, 2008) and others (Jonas et al. 1997; Glitsch et al. 2002; Fulceri et al. 1991) have used to reliably deplete mitochondrial Ca\(^{2+}\). However, because FCCP collapses mitochondrial membrane potential and releases Ca\(^{2+}\) in a concentration-dependent fashion (Heytler & Prichard, 1962), I expected that the FCCP-induced current would have a similar concentration dependency. To test this, FCCP was applied at 30 nM, 300 nM, 1 μM, 3 μM, and 20 μM to cultured bag cell neurons, whole-cell voltage-clamped at a holding potential of -60 mV (Fig. 11A). The concentration-response curve revealed a half-maximal effective concentration of approximately 1 μM and a lack of co-operativity, indicated by a Hill co-efficient of 1.1 (Fig. 11B). Furthermore, the curve began to plateau at around 3 μM making 20 μM FCCP well within
Figure 10. FCCP, but not CPA, causes an inward current

**A**, Whole-cell, voltage-clamp recordings from separate neurons at -60 mV demonstrate that 20 μM FCCP (upper) elicits an inward current, but 20 μM CPA (lower) does not.

**B**, Summary data of the mean peak current normalized to cell size (pA/pF) shows that the current triggered by FCCP is significantly greater than the current produced by either CPA or the vehicle, DMSO. Furthermore, pretreatment with CPA does not significantly change the current induced by FCCP (Kruskal-Wallis nonparametric ANOVA; Dunn’s multiple comparisons test).
Figure 11. The current induced by FCCP is concentration-dependent

**A**, Whole-cell, voltage-clamp recordings from different bag cell neurons in response to 30 nM (n=5), 300 nM (n=5), 1 μM (n=5), 3 μM (n=5), and 20 μM (n=7) FCCP, at a holding potential of -60 mV.

**B**, The curve for FCCP reveals a half-maximal effective concentration (EC₅₀) near 1 μM, a plateau around 10 μM, and a lack of co-operativity with a Hill coefficient of 1. Only one neuron is exposed to a given concentration of FCCP as per the n-values given in A.
the top of the curve.

**The FCCP-induced current is dependent on intracellular Ca\(^{2+}\)**

Depletion of mitochondrial Ca\(^{2+}\) into the cytosol appears to be critical for triggering of the current, raising the possibility it is Ca\(^{2+}\)-activated. To test this, I dialyzed cultured bag cell neurons for 10 min with either regular (5 mM; n=7) or high (20 mM; n=7) EGTA intracellular solution in the pipette, and recorded the FCCP-induced current in whole-cell voltage-clamp at -60 mV. If the current was dependent on intracellular Ca\(^{2+}\) elevation, the high-EGTA should buffer the Ca\(^{2+}\) released from mitochondria and inhibit the response. The FCCP-induced current was attenuated by nearly two-thirds with a high concentration of EGTA (Fig. 12A). This decrease represented a significant reduction in the amplitude of the current compared with regular internal (Fig. 12B).

**Other mitochondrial disruptors fail to evoke a current in bag cell neurons**

CCCP is a reported protonophore uncoupler that is very close in chemical composition to FCCP; however, it is approximately ten times less effective (Heytler & Prichard, 1962). Presumably, CCCP would also deplete the mitochondria of Ca\(^{2+}\) and induce a current. However, in cultured bag cell neurons under whole-cell voltage-clamp, 20 μM FCCP (n=5) evoked an approximately 1 pA/pF inward current, while 20 μM CCCP (n=5) did not (Fig. 13A). The difference in amplitude of the current produced by FCCP and CCCP readily reached significance (Fig. 13B). To account for the dissimilar effect on current, I next used ratiometric imaging of fura PE3-injected cultured bag cell neurons to test whether FCCP or CCCP differentially altered intracellular Ca\(^{2+}\). With external Ca\(^{2+}\) removed, 20 μM FCCP (n=19) caused a rapid and prominent elevation in cytosolic Ca\(^{2+}\) in comparison with 20 μM CCCP (n=14), which did not appreciably alter cytosolic Ca\(^{2+}\) (Fig. 13C). This result was clearly significant (Fig. 13D) and suggests that an inability to deplete Ca\(^{2+}\) from the mitochondria renders CCCP ineffective in eliciting the current.

I subsequently attempted to confirm that the current induced by FCCP is activated by
Figure 12. **Buffering intracellular Ca^{2+} decreases the amplitude of the FCCP-induced current**

**A,** Whole-cell, voltage-clamp recordings from two different neurons at a holding potential of -60 mV show that the current produced by FCCP in the presence of regular EGTA (left) is inhibited when intracellular Ca^{2+} is buffered by a high concentration of EGTA (20 mM; right) in the pipette. In both cases, the neurons are dialysed for 10 min prior to the addition of FCCP. The delay in current onset is not characteristic of high-EGTA and is found throughout both data sets.

**B,** Summary data shows that the mean peak current, normalized to cell size, elicited by 20 μM FCCP is significantly reduced when regular internal is replaced with high-EGTA internal (two-tailed unpaired t-test).
Figure 13. The FCCP-induced current coincides with an increase in intracellular Ca\textsuperscript{2+}

A, Whole-cell, voltage-clamp recordings at -60 mV from separate neurons show an inward current elicited by 20 \(\mu\)M FCCP (upper), but not by 20 \(\mu\)M CCCP (lower) - a drug similar to FCCP in its chemical composition.

B, Summary data of the mean peak current normalized to cell size shows that a significantly greater current is induced by FCCP than by CCCP (two-tailed unpaired t-test; Welch corrected).

C, Ratiometric imaging of intracellular Ca\textsuperscript{2+} in fura PE3-injected bag cell neurons. In Ca\textsuperscript{2+}-free external (cfASW), FCCP depletes the mitochondria of Ca\textsuperscript{2+} and causes an elevation in cytosolic Ca\textsuperscript{2+} (upper), as indicated by an increase in the intensity of the 340/380 ratio; however, in a different neuron, CCCP does not (lower; this trace was recorded by Julia Geiger).

D, Summary data of the mean change in 340/380 shows that the effect of FCCP readily meets significance compared with CCCP (one-tailed unpaired t-test; Welch corrected; the CCCP data was collected by Julia Geiger).
mitochondrial Ca\(^{2+}\), as opposed to an effect of uncoupling the respiratory chain or preventing ATP synthesis. Unfortunately, the mitochondrial respiratory chain blocker, antimycin A (Thorn, 1956), was poorly soluble, potentially due to the high salt content of external solutions, and could not be tested. On the other hand, the mitochondrial ATPase inhibitor, oligomycin A (Lardy \textit{et al.} 1958; Fluharty & Sanadi, 1963), was soluble up to 5 \(\mu\)g/ml and caused a modest elevation in intracellular Ca\(^{2+}\) (n=27) compared with a robust increase induced by FCCP (n=12) (Fig. 14A). The average rise in intracellular Ca\(^{2+}\) observed in response to oligomycin A was significantly lower than that of FCCP, with the onset taking approximately twice as long and the peak taking about five times as long (Fig. 14B). Furthermore, when FCCP was added after oligomycin A had elevated Ca\(^{2+}\) (n=10), this did not significantly affect the amplitude of the Ca\(^{2+}\) increase elicited by FCCP (Fig. 14B). Since oligomycin A released Ca\(^{2+}\), although the time course of release was slower, I tested if it could induce current. However, in neurons whole-cell voltage-clamped at -60 mV in nASW, 20 \(\mu\)M FCCP (n=6) consistently induced an inward current, while oligomycin A (n=6) failed both to evoke current itself and to alter the FCCP-induced current (n=6) (Fig. 15A). There was no significant change in the current produced by FCCP after pretreatment with oligomycin A; however, there was a significant difference between the average amplitude of the current generated by FCCP and oligomycin A (Fig. 15B). This could reflect that specific timing of mitochondrial Ca\(^{2+}\) release is critical in activation of the current, or that oligomycin A liberates Ca\(^{2+}\) from another source.

\textbf{Bafilomycin A does not induce a current}

In addition to the mitochondria and the endoplasmic reticulum, the acidic store (vesicles, lysosomes) contains Ca\(^{2+}\) (Goncalves \textit{et al.} 1999; Christensen, 2002). These organelles accumulate Ca\(^{2+}\) using their H\(^+\) gradient and H\(^+\)/Ca\(^{2+}\) exchange. As a protonophore, FCCP not only has the potential to release Ca\(^{2+}\) by collapsing the H\(^+\) gradient of the mitochondria, but of the acidic store as well. To examine this, cultured bag cell neurons were injected with fura PE3 for Ca\(^{2+}\) imaging. In Ca\(^{2+}\)-free external, 20 \(\mu\)M FCCP caused a marked elevation of intracellular Ca\(^{2+}\)
Figure 14. **Blocking the mitochondrial ATP synthetase slowly increases intracellular Ca^{2+}**

**A**, Intracellular Ca^{2+} is measured using ratiometric imaging in two cultured bag cell neurons injected with fura PE3. Without Ca^{2+} in the extracellular saline (cfASW), 20 μM FCCP produces a sharp elevation in cytosolic Ca^{2+} (*left*). A slower rise in Ca^{2+} about one-third the size of FCCP is seen in response to 5 μg/ml oligomycin A, a blocker of the mitochondrial ATP synthetase (*right*). Pretreatment with oligomycin A does not alter the Ca^{2+} elevation evoked by FCCP. Note that the time base in the right panel is three times that of the left panel.

**B**, Summary data of the mean change in 340/380 reveals that oligomycin A evokes an increase in cytosolic Ca^{2+} that is significantly less than that elicited by FCCP; as well, treatment with oligomycin A does not significantly affect the FCCP-induced intracellular Ca^{2+} increase (Kruskal-Wallis nonparametric ANOVA; Dunn’s multiple comparisons test).
Figure 15. **Blocking the mitochondrial ATP synthetase neither produces a current nor influences the FCCP-induced current**

**A,** Whole-cell, voltage-clamp recordings at -60 mV demonstrate that an inward current is brought about by 20 μM FCCP (*upper*), but not by 5 μg/ml oligomycin A (*middle*); in addition, the FCCP-induced current is not sensitive to oligomycin A pretreatment (*lower*). The upper trace is from a different neuron than the middle and lower traces, which are from the same neuron.

**B,** Summary data of the mean peak current normalized to cell size details that the difference between the current generated by FCCP and oligomycin A readily reaches significance; however, comparison of the current triggered by FCCP following oligomycin A pretreatment with FCCP alone reveals no significant difference (Kruskal-Wallis nonparametric ANOVA; Dunn’s multiple comparisons test).
that peaked within minutes (n=13). Under identical conditions, Ca\(^{2+}\) was depleted from the acidic stores with 100 nM of the H\(^{-}\)-ATPase inhibitor, bafilomycin A (n=7) (Bowman et al. 1988), which elicited a more gradual increase in intracellular Ca\(^{2+}\) and plateaued at an amplitude that was just 15% of FCCP (Fig. 16A). FCCP triggered a significantly greater peak change in 340/380 compared with bafilomycin A (Fig. 16B). Additionally, I examined if prior depletion of Ca\(^{2+}\) from the acidic stores would alter the subsequent rise in Ca\(^{2+}\) caused by FCCP. As shown in the latter part of figure 16 (right), following 20 min of treatment with bafilomycin A, the increase in intracellular Ca\(^{2+}\) evoked by FCCP was largely the same compared to FCCP alone (n=7) (Fig. 16B).

If FCCP is capable of depleting Ca\(^{2+}\) from both acidic and mitochondrial stores, it is possible that acidic store Ca\(^{2+}\) contributes to the FCCP-induced current. However, in cultured bag cell neurons whole-cell voltage-clamped at -60 mV, an inward current was elicited by 20 \(\mu\)M FCCP (n=6), but not by 100 nM bafilomycin A (n=5) (Fig. 17A). The amplitude of the current produced by FCCP was significantly greater compared with bafilomycin A (Fig. 17B). Moreover, prior depletion of bafilomycin A-sensitive stores did not significantly alter the FCCP-induced current (n=5) (Fig. 17A, B), suggesting that the current is not dependent on Ca\(^{2+}\) from acidic stores.

**An alkylation agent attenuates the effect of FCCP on both current and intracellular Ca\(^{2+}\)**

Two primary ways for Ca\(^{2+}\) to leave the mitochondria following loss of membrane potential are through the mitochondrial Ca\(^{2+}\) uniporter or the mitochondrial permeability transitional pore (Campanella et al. 2004). My previous findings suggested that the FCCP-induced current was dependent upon mitochondrial Ca\(^{2+}\). If the release of Ca\(^{2+}\) from the mitochondria can be prevented by blocking the uniporter or the transitional pore, then the current initiated by FCCP should also be reduced. To block the uniporter, cultured bag cell neurons were dialysed for 30 min with intracellular saline containing 100 \(\mu\)M ruthenium red before applying 20 \(\mu\)M FCCP. Ruthenium red has been used to block the uniporter in both Ca\(^{2+}\) imaging as well as
Figure 16. Ca\(^{2+}\) is depleted rapidly from mitochondrial stores, but gradually from acidic stores

A, In the absence of extracellular Ca\(^{2+}\), ratiometric imaging of two separate fura PE3-injected neurons demonstrates that depletion of FCCP-sensitive stores causes a quick elevation in cytosolic Ca\(^{2+}\) (left), compared with the slow rise seen with depletion of stores sensitive to the H\(^+\)-ATPase inhibitor, bafilomycin A (right). In addition, a neuron pretreated with 100 nM bafilomycin A for 20 min presents essentially the same elevation in intracellular Ca\(^{2+}\) due to 20 μM FCCP. Note that the time base in the right panel is three times that of the left panel.

B, Summary data of the mean change in 340/380 indicates that the increase produced by FCCP is significantly greater compared with bafilomycin A. Furthermore, while the average response elicited by FCCP with prior application of bafilomycin A appears smaller, it does not reach significance (Kruskal-Wallis nonparametric ANOVA; Dunn’s multiple comparisons test).
Figure 17. Bafilomycin A does not induce a current and fails to alter the FCCP-induced inward current
A, Whole-cell, voltage-clamp recordings at -60 mV show that depleting Ca$^{2+}$ from mitochondrial stores with 20 μM FCCP elicits an inward current (upper), while depleting acidic stores with 100 nM bafilomycin A does not (middle). Furthermore, the FCCP current persists despite prior depletion with bafilomycin A (lower). Both the middle and lower traces are from the same neuron. The delay in the onset of the FCCP-induced current following bafilomycin A is not consistently observed in other neurons.

B, Summary data of the mean peak current normalized to cell size indicates that FCCP produces a significantly greater current than bafilomycin A (baf), but the reduction of the average FCCP-induced current following 20 min pretreatment with bafilomycin A is not significant despite the apparent lower mean (Kruskal-Wallis nonparametric ANOVA; Dunn’s multiple comparisons test).
patch-clamp experiments involving mitochondrial function (Kirichok et al. 2004; Matlib et al. 1998). The amplitude of the current triggered by FCCP was unaffected by ruthenium red (control = -0.574 ± 0.088 pA/pF, n=6 vs ruthenium red = -0.585 ± 0.049 pA/pF, n=5; p >0.05, one-tailed unpaired t-test). I also explored the role of the Ca\(^{2+}\) released through the transition pore by delivering 20 μM FCCP to cultured bag cell neurons that had been pretreated for 30 min with either ethanol or 100 μM NEM. As an alkylating agent, N-ethylmaleimide (NEM) has a number of potential effects, including inhibition of mitochondrial permeability transition (Petronilli et al. 1994; Jordani et al. 2000; Constantini et al. 1996). Exposure to NEM lowered the current amplitude, but not the time course, by half (Fig. 18A). On average, the current was significantly reduced following treatment with NEM, compared with ethanol (Fig. 18B). Another way to examine the effect of Ca\(^{2+}\) released from the mitochondrial permeability transition pore is to block its formation using cyclosporin A, however, the drug was insoluble in seawater.

It is important to consider that attenuation of the current by NEM could be due to block of mitochondrial Ca\(^{2+}\) release or the plasma membrane channel itself. To verify that NEM was directly reducing Ca\(^{2+}\) release from FCCP-sensitive stores, intracellular Ca\(^{2+}\) was monitored in cultured bag cell neurons. In cfASW, the elevation in cytosolic Ca\(^{2+}\) evoked by 20 μM FCCP was considerably diminished following 30 min treatment with 100 μM NEM (n=10) compared with ethanol (n=9) (Fig. 18C). The mean rise in intracellular Ca\(^{2+}\) triggered by FCCP was lowered significantly in the presence of NEM (Fig. 18D). In summary, NEM caused a notable reduction in both the FCCP-induced current and Ca\(^{2+}\) change, suggesting the current is sensitive to mitochondrial Ca\(^{2+}\).

**The FCCP-induced current is consistent with opening of a voltage-independent cation channel**

To characterize the channel responsible for the FCCP-induced current, I examined reversal potential and membrane conductance under control conditions and once the FCCP-
Figure 18. An alkylating agent attenuates both the rise in intracellular Ca\textsuperscript{2+} and the current induced by FCCP

A, Whole-cell, voltage clamp recordings at -60 mV, in separate neurons bathed in nASW, demonstrate a notable reduction in the amplitude of the FCCP-induced current following a 30 min pretreatment with 100 μM N-ethylmaleimide (NEM; right) but not ethanol (EtOH; left).

B, Summary data of mean peak current normalized to cell size indicates that the current evoked by FCCP is significantly decreased subsequent to treatment with NEM vs ethanol (two-tailed unpaired t-test).

C, Monitoring intracellular Ca\textsuperscript{2+} with ratiometric imaging in Ca\textsuperscript{2+}-free external shows that FCCP causes a prominent rise in Ca\textsuperscript{2+} in ethanol (left); however, this effect is greatly diminished following a 30 min treatment with NEM (right). Note that application of ethanol or NEM alone does not alter intracellular Ca\textsuperscript{2+}.

D, Summary data shows that the mean change in 340/380 evoked by FCCP is significantly less following NEM vs ethanol (two-tailed unpaired t-test; Welch corrected).
induced current had reached a peak. To do this, a 200-ms step from -60 to -70 mV followed by a
10-s ramp from -120 to 0 mV were delivered. Changes in membrane conductance were calculated
from the current during the step. The difference between the current evoked by the ramp before
and at peak response was taken to be the difference current. The whole-cell current reversal
potential was derived from the point where the difference current crossed the abscissa. During the
FCCP-induced current, whole cell conductance increased by over 20 times, which readily met the
level of significance compared with control (n=9) suggesting the opening of a channel (Fig. 19A).
In nASW (n=12), the current-voltage relationship was largely voltage-independent and reversed
around -40 mV, which is characteristic of a nonselective cation channel (Colquhoun et al. 1981;
Partridge & Swandulla, 1988; Hung & Magoski, 2007). Some cation channels are Ca\(^{2+}\)-permeable
(Chesnoy-Marchais, 1985; Jean et al. 2002) and when Ca\(^{2+}\) was removed from the external
solution (n=5), the current became completely linear and the reversal shifted to approximately -47
mV, implying that Ca\(^{2+}\) passes through the channel (Fig. 19B, C). To confirm the Ca\(^{2+}\)
permeability, I examined the FCCP-induced current at -60 mV only. Without extracellular Ca\(^{2+}\),
the amplitude of the FCCP-induced current was significantly diminished (Fig. 19C).

Additionally, I tested if the frequently used cation channel blocker, Gd\(^{3+}\) (Chakfe &
Bourque, 2000; Franco & Lansman, 1990; Popp et al. 1993; Yang & Sachs, 1989), or an inhibitor
of store-operated Ca\(^{2+}\) influx, SKF-96365 (Cabello & Schilling, 1993; Kachoei et al. 2006), could
prevent the opening of a nonselective cation channel by FCCP. However, the average amplitude
of the FCCP-induced current was not significantly altered in the presence of either 100 μM Gd\(^{3+}\)
(control = -1.21 ± 0.19 pA/pF, n=6 vs Gd\(^{3+}\) = -1.24 ± 0.17 pA/pF, n=6; p >0.05, one-tailed
unpaired t-test) or 20 μM SKF-96365 (control = -1.00 ± 0.24 nA/nF, n=5 vs SKF-96365 = -1.23 ±
0.14 pA/pF, n=6; p >0.05, one-tailed unpaired t-test).

**The FCCP-induced current and depolarization are not sensitive to Ni\(^{2+}\)**

The depolarization evoked by FCCP in nASW typically reached levels between -20 and -
30 mV (see Fig. 9). With that stated, the FCCP-induced current reversed at -40 mV. What could
Figure 19. The FCCP-induced current is consistent with the opening of a voltage-independent cation channel

A, Left, Whole-cell, voltage-clamp recording of current during a 10 mV hyperpolarizing step from a holding potential of -60 mV before (thin line) and after (thick line) 20 μM FCCP indicates a marked elevation in conductance. Right, Summary data shows that FCCP significantly increases conductance compared with control (two-tailed paired t-test).

B, Subtraction current (FCCP minus control) during a ramp from -120 to 0 mV (see inset). In nASW (thick line), the FCCP-induced current is largely linear and reverses near -40 mV; whereas in Ca2+-free external (thin line), the reversal is left-shifted.

C, Left, Consistent with Ca2+ permeability, the removal of extracellular Ca2+ significantly shifts the mean reversal potential from approximately -40 mV to -47 mV (two-tailed unpaired t-test). Right, Summary data of the mean peak current normalized to cell size shows that this also significantly decreases the amplitude of the current induced by FCCP (two-tailed unpaired t-test).
account for the additional degree of depolarization? An obvious example is the Ni\(^{2+}\)-sensitive persistent Ca\(^{2+}\) current, which can be triggered in bag cell neurons by modest depolarization (Tam & Magoski, 2007). I confirmed that Ni\(^{2+}\) does not block the FCCP-induced current by pretreating whole-cell voltage-clamp cultured bag cell neurons with 10 mM Ni\(^{2+}\) before applying 20 \(\mu M\) FCCP. There was no significant difference in the current in the presence (n=6) or absence (n=6) of Ni\(^{2+}\). Next, whether Ni\(^{2+}\) had any effect on the depolarization evoked by FCCP was examined in sharp-electrode current-clamp. The spiking that occasionally coincided with the FCCP-induced depolarization was consistently abolished following Ni\(^{2+}\) pretreatment, which is not surprising since Ca\(^{2+}\) channels are largely responsible for the rising phase of the action potential in bag cell neurons (DeReimer et al. 1985). However, pretreatment with Ni\(^{2+}\) (n=5) did not significantly alter the FCCP-induced depolarization (n=18) (Fig. 20A, B). These observations provide evidence against the involvement of Ca\(^{2+}\) channels in the depolarization elicited by FCCP.
Figure 20. The FCCP-induced current and depolarization are not blocked by Ni$^{2+}$.

A, Whole-cell, voltage-clamp recordings at -60 mV of separate neurons, with and without 5 min of 10 mM Ni$^{2+}$ pretreatment, indicate that Ni$^{2+}$ does not alter the amplitude of the FCCP-induced current.

B, Summary data shows no significant difference in the mean peak current, normalized to cell size, elicited by 20 μM FCCP in the presence or absence of Ni$^{2+}$ (one-tailed unpaired t-test).

C, Sharp-electrode current-clamp recordings in separate neurons show that the FCCP-induced depolarization is not altered by 5 min of 10 mM Ni$^{2+}$ pretreatment. The firing of action potentials in response to 20 μM FCCP is observed in 8 out of the 18 neurons under control conditions; however, this response is consistently eliminated in all 5 neurons previously exposed to Ni$^{2+}$.

D, Summary data of the mean change in membrane potential shows that the depolarization induced by FCCP is not significantly different, with or without Ni$^{2+}$ pretreatment (one-tailed unpaired t-test).
Chapter 4: Discussion

The capacitance change is consistent with neurosecretion

Since its first use in 1982 by Neher and Marty, capacitance tracking has become a well-established means of monitoring the release of both classical and peptide neurotransmitter (Lim et al. 1990; Neves et al. 2001; Klyachko & Jackson, 2002; Hull & von Gersdorff, 2004). The fusion of vesicles with the plasma membrane, expels their contents into the extracellular space by exocytosis (del Castillo & Katz, 1955; Katz & Miledi, 1967 a,b; Heuser et al. 1979; Rizzoli & Betz, 2004). As vesicles fuse, this causes an increase in membrane surface area, which can be reliably detected by capacitance measurements.

Ca2+ entry is necessary for triggering neurosecretion (Katz & Miledi, 1967a). Although capable of entry through Ca2+ channels, Ba2+ substitutes poorly if at all for Ca2+ in neurotransmitter release (Miledi et al. 1966; Kilic et al. 2001; Shin et al. 2003). Recordings from isolated rat neurohypophysis terminals showed that even when Ba2+ currents were larger than Ca2+ currents during depolarization, Ba2+ entry evoked much less secretion (Nowycky et al. 1998). In bag cell neurons, the capacitance increase evoked by the train was absent when external Ca2+ was replaced with Ba2+. This is consistent with an ineffectiveness of Ba2+ at initiating secretion.

Peptide release can be eliminated by removing external Ca2+ or blocking voltage-gated Ca2+ channels, demonstrating an absolute requirement for Ca2+ influx from the extracellular space (Hsu & Jackson, 1996; Whim et al. 1997; Branchaw et al. 1998; Sedej et al. 2004; Soldo et al. 2004). Ni2+ is a non-selective, but very effective Ca2+ channel blocker at mM doses (Byerly et al. 1985; McFarlane & Gilly, 1998; Hung & Magoski, 2007). Pretreating bag cell neurons with Ni2+ abolished the capacitance increase observed in response to the train, which is in direct agreement with the block of voltage-gated Ca2+ channels preventing peptide secretion.

The molecular events that are responsible for neurosecretion involve interaction of specific vesicle- and membrane-associated proteins in a stable, low-energy SNARE complex;
which is subsequently dismantled into recyclable components, by the ATPase, NSF, once fusion is complete (Jessell & Kandel, 1993; Rettig & Neher, 2002; Smith et al. 2008). N-ethylmaleimide (NEM) hinders peptide secretion, presumably by alkylating NSF (Block et al. 1988; Chavez et al. 1996; Han et al. 1999). In the present study, exposure of bag cell neurons to NEM reduced the capacitance increase evoked by the train. However, caution must be taken when stating that the effect of NEM on the capacitance change is attributable to NSF alkylation. Work on identified Aplysia neurons L3, L6, and R15 suggests that NEM is capable of interfering with the deactivation of a G-protein that inhibits Ca\(^{2+}\) currents (Fryer, 1992). Thus, altering the Ca\(^{2+}\) current by NEM could attenuate the secretory response. On the contrary, NEM did not significantly reduce the Ca\(^{2+}\) current in bag cell neurons. Furthermore, any NEM-induced change to Ca\(^{2+}\) current that may have occurred would have unlikely influenced secretion. Hung and Magoski (2007) found that a 50 ms pulse duration during a 5 Hz train produces about 50% less Ca\(^{2+}\) influx compared with 75 ms, yet I did not observe a significant difference in the capacitance increase evoked using either of these pulse durations. It does not seem probable that the approximate 70% drop in apparent secretion when bag cells are treated with NEM is due to a smaller Ca\(^{2+}\) current.

The capacitance change I observed in cultured bag cell neurons is consistent with neuropeptide release. This is supported by previous experiments that directly detected ELH secretion, using radioimmunoassay or high-pressure liquid chromatography, in response to an afterdischarge or a high-K\(^+\) stimulus in bag cell neurons from an isolated abdominal ganglion (Michel & Wayne, 2002), isolated bag cell neuron clusters (Loechner et al. 1990), or isolated bag cell neuron neurites (Lee & Wayne, 2004). Furthermore, employing mass spectrometry, Jo et al. (2007) showed that even a few or single cultured bag cell neurons release peptides subsequent to administration of high K\(^+\). Thus, the capacity to secrete is maintained in culture, which is the preparation used in the present study.
A slow rate, high threshold secretory process in bag cell neurons

At the mouth of open Ca$^{2+}$ channels are small regions of concentrated Ca$^{2+}$ called microdomains that are strategically located for initiating fusion of nearby vesicles (Llinas et al. 1992; Neher, 1998; Oré & Artalejo, 2004; Neher & Sakaba, 2008). Classical neurotransmitter release is rapid, between microseconds to a few milliseconds, with a requirement of high local Ca$^{2+}$ concentration provided by the close proximity of Ca$^{2+}$ channel and vesicle (Llinas et al. 1981; von Gersdorff & Matthews, 1994; Neher, 1998). Because of this close interaction, classical release is often sensitive to BAPTA, but typically insensitive to EGTA. Even though they have a similar affinity for Ca$^{2+}$, unlike BAPTA, EGTA is not fast enough to bind Ca$^{2+}$ over such short distances (Tsien, 1980; Smith et al. 1984; Naraghi, 1997).

Neuroendocrine release is typically slower, occurring between a few milliseconds to seconds, requires low to high Ca$^{2+}$ concentrations, and it is often sensitive to EGTA - implying the vesicles are loosely coupled to Ca$^{2+}$ channels. There are many examples of neuroendocrine and peptide release that display some or all of these characteristics, including catecholamines (Neher & Marty, 1982), proopiomelanocortin (Thomas et al. 1993; Sedej et al. 2004), atrial natriuretic factor (Burke et al. 1997), insulin (Barg et al. 2002), growth hormone (Kilic et al. 2001), and oxytocin and vasopressin (Lim et al. 1990; Branchaw et al. 1998; Nowycky et al. 1998; Soldo et al. 2004).

Similar to atrial natriuretic factor (Burke et al. 1997), growth hormone (Kilic et al. 2001), and proopiomelanocortin (Sedej et al. 2004), secretion from bag cell neurons is slower to develop, dependent upon prominent Ca$^{2+}$ influx, and reduced by EGTA. In some neurons, there exists a notable delay between Ca$^{2+}$ influx and secretion. For example, in oxytocin- and vasopressin-secreting nerve terminals from the neurohypophysis, Nowycky et al. (1998) postulate that a Ca$^{2+}$-dependent preparatory step must occur before further Ca$^{2+}$ influx can trigger exocytosis. The final step of exocytosis is evoked by a high concentration of Ca$^{2+}$ that surpasses a certain threshold. The slow onset and high-threshold of Ca$^{2+}$ needed for secretion from bag cell neurons could
reflect a Ca^{2+}-dependent priming step. A slow, high-threshold mechanism seems appropriate, as the animal must fully commit to the energy-demanding and highly vulnerable behaviour of laying eggs.

**Depletion and recovery of the readily releasable pool**

Once vesicles in the readily releasable pool have been depleted, they are often quickly replaced with vesicles from the reserve pool, such that most peptide release shows modest to no depletion with repeated stimulation (Thomas *et al.* 1993; Lim *et al.* 1990; Hsu & Jackson, 1996; Nowycky *et al.* 1998; Kilic *et al.* 2001; Sedej *et al.* 2004; Soldo *et al.* 2004). Peptidergic *Aplysia* motor neurons B1 and B2 exhibit little depletion following repeated stimulation (Whim *et al.* 1997). However, bag cell neuron repletion is slow and the capacitance response is reduced by about half with a second stimulus. ELH-containing vesicles are large, with a mean diameter of approximately 150 nM (Fisher *et al.* 1988), and my finding could reflect the limited ability of large vesicles in the reserve pool to rapidly replenish the releasable pool. Burke *et al.* (1997) suggest that once the releasable pool is depleted, sustained secretion is restricted to a small proportion of mobile granules and how slowly they diffuse through the cell. Another potential rate-limiting factor is lengthy vesicle emptying, since it would take longer for vesicles to become available for recycling. According to Barg *et al.* (2002), when a peptidergic vesicle fuses, it creates a fusion pore not large enough for high molecular weight proteins to exit until the pore has expanded or the vesicle has fused entirely. Susceptibility to run down may again be due to the all-or-none nature of bag cell neuron function. Finally, the depletion I observed may not be entirely representative of bag cell neuron secretion since my recordings were done at the soma, which is not the primary source of secretion *in vivo* and may not be as capable of quick replenishment. However, Pow & Morris (1989) first demonstrated that oxytocin and vasopressin neurons release peptide from the soma as well as dendrites.

Exocytosis is immediately followed by retrieval of the membrane by endocytosis, which is reflected by a decrease in capacitance. Endocytosis can take place *via* a clathrin-dependent or -
independent process (Kilic et al. 2001; Yamashita et al. 2005; Smith et al. 2008). The clathrin-dependent mode is slower, and relies on the binding of dynamin and GTP hydrolysis. Further investigation is needed to determine the nature of endocytosis in bag cell neurons. However, based on the delayed recovery of the readily releasable pool, slow clathrin-dependent endocytosis may predominate. The present study shows similarity with reports of bulk endocytosis after prolonged secretion, where additional membrane, along with the fused vesicles, is retrieved to an extent that the post-secretion surface area is less than before exocytosis (Engisch & Nowycky, 1998; Hsu & Jackson, 1996; Smith et al. 2008).

**Store-operated secretion**

For Ca$^{2+}$ from an intracellular store to actively contribute to secretion is rare. Yet, there are examples where Ca$^{2+}$ liberated from the endoplasmic reticulum, either via metabotropic receptors or through Ca$^{2+}$-induced Ca$^{2+}$-release, potentiates or causes secretion of classical and peptide transmitter. This includes potentiating GABA release during associative learning in *Hermissenda* (Blackwell & Alkon, 1999), as well as acetylcholine in *Aplysia* neurons (Chameau et al. 2001), oxytocin from the dendrites of supraoptic nucleus neurons (Ludwig et al. 2002), and atrial natriuretic factor from *Drosophila* motor neurons (Shakiryanova et al. 2007). Endoplasmic reticulum Ca$^{2+}$ has been found to directly cause secretion of luteinizing hormone from gonadotrophs (Tse et al. 1997), serotonin from leech *Retzius* neurons (Trueta et al. 2004), GABA from chick amacrine cells (Warrier et al. 2005), glutamate from salamander photoreceptors (Suryanarayanan & Slaughter, 2006) and catecholamines from rat chromaffin cells (Miranda-Ferreira et al. 2009). In the present study, it appears that endoplasmic reticulum Ca$^{2+}$ does not trigger secretion, since treating bag cell neurons with CPA had no effect on membrane capacitance. This result is consistent with Jonas et al. (1997) where Ca$^{2+}$ released from the endoplasmic reticulum by thapsigargin, which acts analogously to CPA, does not induce ELH release from bag cell neurons. Additionally, endoplasmic reticulum Ca$^{2+}$ does not induce the
secretion of serotonin from rat prostate neuroendocrine cells (Kim et al. 2004) or insulin from rat beta cells (Gilbert et al. 2008).

The predominant role of the mitochondria in transmitter release is to buffer Ca\(^{2+}\) influx. For example, the clearance by the mitochondria limits exocytosis from bovine chromaffin cells (Giovannucci et al. 1999). However, in the synaptic terminal of bipolar cells, mitochondria do little to remove Ca\(^{2+}\), but are fundamental in providing the energy for Ca\(^{2+}\) clearance (Zenisek & Matthews, 2000). Zhong et al. (2001) suggest a major contribution by the mitochondria in regulating presynaptic Ca\(^{2+}\) during and after tetanic stimulation at the crayfish neuromuscular junction. Finally, the buffering action of mitochondria at central glutamatergic terminals reduces short-term presynaptic depression (Billups & Forsythe, 2002). In none of these cases does mitochondrial Ca\(^{2+}\) evoke release of transmitter itself. However, bag cell neurons appear unique in that release of mitochondrial Ca\(^{2+}\) causes apparent peptide secretion. The exception is that FCCP can elicit catecholamine-release from chromaffin cells (Miranda-Ferreira et al. 2009).

**Mitochondrial Ca\(^{2+}\) specifically opens a membrane channel**

The present study suggests that mitochondrial Ca\(^{2+}\), but not endoplasmic reticulum or acidic store Ca\(^{2+}\), preferentially opens a membrane channel. CPA depletes Ca\(^{2+}\) from the endoplasmic reticulum (Seidler et al. 1989; Tu et al. 2006); however, CPA did not evoke a current in cultured bag cell neurons. The acidic stores, which include vesicles and lysosomes, sequester Ca\(^{2+}\) via a Ca\(^{2+}/H^+\) antiporter, driven by a H\(^+\) gradient created by a H\(^+\)-ATPase (Goncalves et al. 1999). Bafilomycin A blocks the V-type H\(^+\)-ATPase, which eliminates the electrochemical gradient and results in Ca\(^{2+}\) release (Bowman et al. 1988; Goncalves et al. 1999; Christensen et al. 2002). A protracted and diminutive increase in cytosolic Ca\(^{2+}\) was observed in response to bafilomycin A in bag cell neurons, consistent with a prior report from our laboratory (Kachoei et al. 2006). Yet, similar to the endoplasmic reticulum, acidic store Ca\(^{2+}\) did not elicit any current.

Since collapsing the mitochondrial membrane potential with FCCP could cause a
reduction in ATP levels, this could be causing the current instead of Ca$^{2+}$ release. Oligomycin A blocks the mitochondrial ATP synthetase, which like FCCP, would eliminate ATP production by the mitochondria (Lardy et al. 1958; Fluharty & Sanadi, 1963). In HeLa cells, FCCP caused a gradual depletion of ATP with glucose in the bath (Collins et al. 2000). Similarly, ATP levels drop with oligomycin A in Chinese hamster ovary cells, but much of that effect is rescued by glucose (Cho et al. 1997). Similar to the present study, these cases reported either a small or no change in cytosolic Ca$^{2+}$ to oligomycin A. Although poisoning mitochondria reduces oxygen consumption, it appears that glycolysis is able to provide sufficient levels of ATP in the absence of functional mitochondria in the short term. (Land et al. 1999). Additionally, there was a constant supply of ATP in the whole-cell pipette when I recorded FCCP-induced current.

The mitochondria may not be the source of Ca$^{2+}$ liberated by oligomycin A. Reduced ATP levels could deplete Ca$^{2+}$ from other stores dependent on energy for Ca$^{2+}$ uptake, such as the endoplasmic reticulum Ca$^{2+}$ pump or the V-type H$^+$-ATPase. However, our laboratory has reported that FCCP does not deplete Ca$^{2+}$ from the endoplasmic reticulum (Geiger & Magoski, 2008). Yet, lowered ATP levels may impact the H$^+$-ATPase and cause Ca$^{2+}$ release from vesicles and lysosomes. Finally, oligomycin can inhibit the Na$^+$/K$^+$-ATPase, which would elevate intracellular Na$^+$ and impede the Na$^+$/Ca$^{2+}$ exchanger from removing Ca$^{2+}$ (Fahn et al. 1966; Knox et al. 1996). Regardless of origin, Ca$^{2+}$ liberated by oligomycin does not evoke a current in bag cell neurons, providing further evidence that the FCCP-induced current is not due to changes in ATP levels. In smooth muscle, CCCP alters the amplitude of Ca$^{2+}$ transients and prolongs recovery, while oligomycin does not, consistent with mitochondrial Ca$^{2+}$ handling, rather than cessation of ATP production, being responsible (McCarron & Muir, 1999).

Due to the very negative potential across the mitochondrial membrane, Ca$^{2+}$ enters the mitochondria from the cytosol via the Ca$^{2+}$ uniporter; however, FCCP takes the potential to zero and causes Ca$^{2+}$ to exit (Heytler & Prichard, 1962; Moore, 1971). FCCP induces small Ca$^{2+}$ increases in a number of cell types, such as oligodendrocytes (Simpson & Russell, 1996), HeLa
cells (Collins et al. 2000), endothelia (Park et al. 2002), sympathetic (Friel & Tsien, 1994; Colegrove et al. 2000), hippocampal (Brustovetsky & Dubinsky, 2000), and dorsal root ganglion neurons (Werth & Thayer, 1994; Jackson & Thayer, 2006). In bag cell neurons, depleting the mitochondria of Ca^{2+} with FCCP evokes a large Ca^{2+} elevation (Jonas et al. 1997; the present study), similar to that observed in hepatocytes and A23187 cells (Fulceri et al. 1991), Helisoma motor neuron B5 (Jensen & Rehder, 1991), hippocampal neurons (Nowicky & Duchen, 1998), bipolar neurons (Zenisek & Matthews, 2000), and chromaffin cells (Hernandez-Guijo et al. 2001). Surprisingly, CCCP, a structural analog of FCCP, did not alter bag cell neuron intracellular Ca^{2+} or current. This may be accounted for by CCCP being about tenfold less effective in depolarizing mitochondria (Heytler & Prichard, 1962). Furthermore, CCCP increases basal Ca^{2+} only slightly in dorsal root ganglion neurons (Werth & Thayer, 1994), or not at all in crayfish motor neurons (Tang & Zucker, 1997), guinea pig smooth muscle (McCarron & Muir, 1999), and the Calyx of Held (Kim et al. 2005). However, this may reflect the resting mitochondrial Ca^{2+} in these preparations more than an ineffectiveness of CCCP.

One potential route for Ca^{2+} to leave the mitochondria is through the uniporter, a voltage-independent ion channel in the inner membrane that is blocked by ruthenium red; however, this channel shows extreme inward rectification between -20 and +20 mV, and upon collapse of the membrane potential by FCCP, any efflux though this channel would be eliminated (Moore, 1971; Matlib et al. 1998; Kirichok et al. 2004). Therefore, it is not surprising that dialysing bag cell neurons with ruthenium red did not prevent the FCCP-induced current. Alternatively, Ca^{2+} could be released through the mitochondrial permeability transition pore, an inner mitochondrial membrane, voltage-dependent mega channel that is opened by FCCP-induced mitochondrial depolarization and is blocked by NEM (Hunter & Haworth, 1979; Costantini et al. 1996). NEM may interfere with the vicinal nature of a pair of sulfhydryl groups that are important for pore opening (Petronilli et al. 1994). Despite the potential that NEM could alter sulfhydryl groups of
many proteins, the fact that it attenuated both the FCCP-induced $\text{Ca}^{2+}$ elevation and current is consistent with mitochondrial $\text{Ca}^{2+}$ being the trigger.

Cho et al. (1997) reported that store-operated channels in Chinese hamster ovary cells and Jurkat T-cells are inhibited by mitochondrial depolarization and loss of $\text{Ca}^{2+}$ uptake, yet not by reduced ATP production or transition pore block. This is in contrast with my observations, since FCCP failed to evoke a current in the cell lines; that study also suggests that $\text{Ca}^{2+}$ uptake, rather than $\text{Ca}^{2+}$ release by the mitochondria is responsible for the effect on membrane current. Similarly, IP$_3$ receptors in intact HeLa cells are inhibited by mitochondrial depolarization and this is not prevented by transition pore block (Collins et al. 2000).

**The FCCP-activated current is consistent with a non-selective cation channel**

The FCCP-induced current is a voltage-independent conductance up to the point of reversal, after which some inward rectification is evident. The inward current is accompanied by an increase in conductance and reverses at -40 mV, indicative of the opening of a non-selective cation channel. Typically, a current that reverses between -40 and +20 mV is characteristic of a channel that passes cations with a varying degree of selectivity and no clear preference (Kass et al. 1978; Colquhoun et al. 1981; Partridge & Swandulla, 1988; Partridge et al. 1994). Without external $\text{Ca}^{2+}$, the FCCP-induced current is diminished and the reversal shifted to the left, implying that the channel is $\text{Ca}^{2+}$ permeable.

As $\text{Ca}^{2+}$ appears to elicit the current, one or more bag cell neuron $\text{Ca}^{2+}$-activated channels may be implicated. It is unlikely that the voltage-dependent cation channel, which reverses well above 0 mV, is involved in generating the current itself, although it may contribute to the depolarization through secondary activation (Wilson et al. 1996; Geiger et al. 2009). A more likely candidate is the voltage-independent cation channel, with a reversal potential near -40 mV, triggered by $\text{Ca}^{2+}$ influx (Hung & Magoski, 2007). Thus, it is not surprising that the store-operated channel blocker, SKF-96365, did not alter the FCCP-induced current, since it also does not affect
the voltage-independent cation channel (Hung & Magoski, 2007). A final possibility is the voltage-independent cation channel that is opened by flufenamic acid-mediated Ca\(^{2+}\) release and reverses near -15 mV (Gardam et al. 2008).

Hyllienmark and Brismar (1996) found that inhibition of metabolism with FCCP, dinitrophenol or cyanide hyperpolarizes pyramidal cells of the hippocampus in slice due to opening of ATP-sensitive K\(^+\) channels. Paradoxically, FCCP, cyanide, and low oxygen hyperpolarize dissociated pyramidal cells through the opening of Ca\(^{2+}\)-activated K\(^+\) channels (Nowicky & Duchen, 1998). In locus ceruleus neurons, FCCP, cyanide, or low glucose generate an outward current sensitive to either depletion of endoplasmic reticulum Ca\(^{2+}\) by thapsigargin, or block of Ca\(^{2+}\)-activated K\(^+\) and K\(_{ATP}\) channels (Murai et al. 1997). For these neurons, a reduction in ATP both activates K\(_{ATP}\) channels and indirectly depletes Ca\(^{2+}\) from the ER, which in turn switches on Ca\(^{2+}\)-activated K\(^+\) channels.

**Changes to intracellular pH or proton permeability likely do not underlie the FCCP-induced current**

As a protonophore, FCCP theoretically increases the permeability of H\(^+\) across all membranes, thus allowing H\(^+\) to contribute to the resting potential (Bashford et al. 1985). However, it is unlikely this is responsible for the FCCP-induced current observed in the present study. Under control conditions (pH = 7.3 in and 7.8 out), the H\(^+\) equilibrium potential is -30 mV, and too positive with respect to the reversal potential of the FCCP-induced current. FCCP could lower pH within the cell, as it both drives H\(^+\) into the mitochondria and draws H\(^+\) out of acidic stores as well as the golgi apparatus (Werth & Thayer, 1994; Park et al. 2002). However, if FCCP acidifies the cytosol even by half a pH unit, the equilibrium potential for H\(^+\) would be too negative at -58 mV. Furthermore, if FCCP had a generalized effect on H\(^+\) permeability, this would manifest in all cells, and while FCCP or CCCP depolarize Lettre cells and lymphocytes (Bashford et al. 1985), RBL-1 cells (Mohr & Fewtrell, 1987), and endothelia (Park et al. 2002), they fail to depolarize or produce a current in hamster kidney cells (Bashford et al. 1985), crayfish motor
neurons (Tang & Zucker, 1997), chromaffin cells (Giovannucci et al. 1999; Hernandez-Guijo et al. 2001), hippocampal neurons (Partridge & Valenzuela, 1999), and, incongruently, RBL-1 cells (Glitsch et al. 2002).

An increase in H⁺ permeability would not be sensitive to changes in extracellular Ca²⁺ or intracellular Ca²⁺ buffering; in contrast, the FCCP-induced current in bag cell neurons was altered by both. Park et al. (2002) discovered an FCCP-induced depolarization of aortic endothelia caused by H⁺ and Na⁺ currents that are dependent on the proton gradient across the plasma membrane; however, that depolarization is unaffected by removal of extracellular Ca²⁺, buffering intracellular Ca²⁺, and presents an EC₅₀ of 45 μM. Intracellular acidification could activate a pH-sensitive cation channel; however, in dorsal root ganglion and trigeminal neurons, this channel is blocked by ruthenium red, while the FCCP-induced current is not (Garcia-Hirschfeld et al. 1995; Zeilhofer et al. 1996). Finally, the ineffectiveness of bafilomycin, which would potentially acidify the cytosol, on bag cell neuron holding current, points away from H⁺ as a gating factor.

**Store Ca²⁺ contributes to other cation channel-mediated depolarizations**

Endoplasmic reticulum Ca²⁺ either evokes or potentiates voltage-independent cation channels in a number of neuron types. Ca²⁺-induced Ca²⁺ release potentiates cation channel-dependent depolarizing afterpotentials in both supraoptic (Li & Hatton, 1997) and hippocampal neurons (Partridge & Valenzuela, 1999). Also, metabotropic or CPA-stimulated Ca²⁺ release potentiates supraoptic neuron cation channels (Li et al. 1999), evokes a voltage-independent cation channel in dorsal root ganglion neurons (Crawford et al. 1997), and turns on TrpC1/C5, C3, M4, and M5 cation channels in cell lines (Zitt et al. 1997; Strubing et al. 2001; Launay et al. 2002; Prawitt et al. 2003; Liu & Liman, 2003). Thus, while a store-operated cation channel is not unique, activation of such a current by mitochondrial Ca²⁺ has not been reported until now. However, Partridge and Valenzula (1999) found that the cation channel-dependent depolarizing afterpotentials in supraoptic neurons were potentiated, although not triggered, by mitochondrial Ca²⁺. Because bag cell neurons release mitochondrial Ca²⁺ during prolonged action potential
firing, the cation channel described here may contribute to the depolarization necessary for the afterdischarge, reproductive hormone release, and ovulation.

**The lonely endoplasmic reticulum Ca\(^{2+}\) store**

It appears that mitochondrial Ca\(^{2+}\) preferentially triggers a non-selective cation channel and secretion. However, Ca\(^{2+}\) from the endoplasmic reticulum fails to influence either the activity of this channel or peptide release. This suggests that mitochondria are optimally positioned for accessing the plasma membrane, while the endoplasmic reticulum is not (Fig. 21). Why does endoplasmic reticulum Ca\(^{2+}\) fail to cause secretion or open channels that are sensitive to mitochondrial Ca\(^{2+}\)? The endoplasmic reticulum is sometimes associated more with the nucleus than the membrane, while mitochondria are evenly distributed (Palade, 1955; Verkhratsky, 2005). Furthermore, dye-staining for mitochondria shows that they accumulate in the soma and neurites of bag cell neurons (White & Kaczmarek, 1997). Therefore, differential distribution may give mitochondrial Ca\(^{2+}\) preferential access to membrane ion channels. Jonas *et al.* (1997) demonstrated that spatial distribution of Ca\(^{2+}\) stores contributes to their differing ability to initiate neurosecretion from bag cell neurons. Ca\(^{2+}\) released from an unidentified store by insulin uniformly elevated Ca\(^{2+}\) throughout the soma and neurites, as well as stimulated secretion; while depletion of endoplasmic reticulum Ca\(^{2+}\) raised Ca\(^{2+}\) levels at the soma, but less so in distal neurites, and did not induce release. Furthermore, in oligodendrocytes, groups of mitochondria are localized in areas where there is amplification of the Ca\(^{2+}\) signal during wave propagation (Simpson & Russell, 1996).

Selective dependence could be because the cation channel is gated by an intermediate, yet mobile Ca\(^{2+}\)-sensitive protein, such as calmodulin (CaM). If the protein favours a close association with the mitochondria, as opposed to the endoplasmic reticulum, then Ca\(^{2+}\) released from the mitochondria would be privileged for channel activation. Pardue *et al.* (1981) observed that CaM is highly associated with mitochondria, while Wood *et al.* (1980) used immuno-electron microscopy to show diffuse staining for CaM throughout basal ganglia neurons, with some of the
Figure 21. Bag cell neuron Ca\textsuperscript{2+} dynamics
An updated schematic of the regulation of intracellular Ca\textsuperscript{2+}. Ca\textsuperscript{2+} influx through voltage-gated Ca\textsuperscript{2+} channels (I_{Ca\textsubscript{2+}}) triggers secretion of egg-laying hormone (ELH). Collapsing the mitochondrial membrane potential with FCCP causes depletion of mitochondrial Ca\textsuperscript{2+} stores, which produces secretion and activates a membrane cation channel. Ca\textsuperscript{2+} liberated from the endoplasmic reticulum by the SERCA-blocker, CPA, does not similarly affect secretion or membrane activity. The present study confirms that Ca\textsuperscript{2+} influx causes secretion in bag cell neurons and determines that mitochondrial Ca\textsuperscript{2+} activates both a cation channel and secretion, while endoplasmic reticulum Ca\textsuperscript{2+} does not.
highest labelling concentrated at mitochondria. In an example of local Ca\(^{2+}\) elevation specifically triggering CaM, Deisseroth et al. (1998) show that Ca\(^{2+}\) influx through L-type channels and NMDA receptors, but not N or P/Q-type Ca\(^{2+}\) channels, stimulate CaM translocation to the nucleus. A similar example is found in bag cell neurons, as Ca\(^{2+}\) entry through the voltage-dependent cation channel preferentially causes refractoriness, whereas influx via voltage-dependent Ca\(^{2+}\) channels does not (Magoski et al. 2000). However, I cannot exclude the possibility that the reduced magnitude or rate of the Ca\(^{2+}\) response evoked by CPA, compared with FCCP, could limit the ability of endoplasmic reticulum Ca\(^{2+}\) to induce current in bag cell neurons. Thus, FCCP might be the preferred trigger for this current simply because it produces a more rapid and exaggerated increase in Ca\(^{2+}\).

In light of both the contribution of the endoplasmic reticulum to Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Geiger & Magoski, 2008) and the association of the afterdischarge with increased IP\(_{3}\) levels (Fink et al. 1988), what is the role of endoplasmic reticulum Ca\(^{2+}\) in bag cell neurons? Endoplasmic reticulum Ca\(^{2+}\) could activate Ca\(^{2+}\)-dependent enzymes, which produce important changes to Ca\(^{2+}\) channel function, Ca\(^{2+}\)-induced Ca\(^{2+}\) release, and neurosecretion during the slow phase and following termination of the afterdischarge (DeRiemer et al. 1984, 1985; Loechner et al. 1992; Wayne et al. 1998, 1999; Geiger & Magoski, 2008). Moreover, depletion of endoplasmic reticulum Ca\(^{2+}\) opens a store-operated Ca\(^{2+}\) influx pathway in bag cell neurons, however it is the depletion itself that opens the channel and not the Ca\(^{2+}\) per se (Kachoei et al. 2006).

**Future Directions**

A limitation of using capacitance tracking as an indicator of secretion is that it reflects the processes of both exocytosis and endocytosis, thus representing a net change in membrane surface area. Measuring the release of egg-laying hormone from cultured bag cell neurons would allow for the comparison of my findings using capacitance tracking with a direct measure of secretion. Another potential limitation in the present study could arise from tracking capacitance in the
soma, which may not be the predominant source of secretion in bag cell neurons. It would be interesting to track capacitance in growth cones in response to Ca\textsuperscript{2+} influx through voltage-gated Ca\textsuperscript{2+} channels and compare these results with those observed in the soma.

To examine if there is a differential distribution of mitochondria and endoplasmic reticulum throughout the cell, I would localize mitochondria and endoplasmic reticulum using tracker dyes or electron microscopy. Another way to determine whether there is a spatial distinction between Ca\textsuperscript{2+} released by the mitochondria and by the endoplasmic reticulum would be to image Ca\textsuperscript{2+} locally and compare the patterns of Ca\textsuperscript{2+} elevation in response to FCCP and CPA. As I had alluded to earlier, it is possible that Ca\textsuperscript{2+} is not released in the proximity of the membrane channel at all. Ca\textsuperscript{2+} from the mitochondria may trigger the translocation of a Ca\textsuperscript{2+}-dependent mobile intermediate, such as CaM or protein kinase C (PKC), which could in turn affect the channel. It would provide useful insight to observe the effect of blocking or enhancing the activity of CaM or PKC on the FCCP-induced current.
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