Ca^{2+} influx and release in *Aplysia* bag cell neurons

By

Julia Elizabeth Geiger

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Abstract

Intracellular Ca^{2+} is influenced by extracellular Ca^{2+} influx and release from internal stores. Depending on the initial influx, a range of responses can result. To better understand the processes surrounding Ca^{2+} handling, I examined intracellular Ca^{2+} following receptor-operated or voltage-gated Ca^{2+} influx in bag cell neurons of the marine mollusc, *Aplysia californica*. Following a brief synaptic input, these neuroendocrine cells undergo a 30 min period of firing, known as the afterdischarge, triggering secretion of neuropeptides that initiate egg-laying. I hypothesize that Ca^{2+} influx occurs via receptor-mediated activation of a non-selective cation channel and activity-dependent elevation of Ca^{2+} produces Ca^{2+} release from intracellular stores. To test this, cultured bag cell neurons were injected with Ca^{2+} indicator, fura-PE3, and subjected to simultaneous imaging and electrophysiology.

Pharmacological activation of a non-selective cation channel that drives the afterdischarge, produced inward current and, as assessed by Mn^{2+}-quench of fura, Ca^{2+} entry. This receptor-operated Ca^{2+} influx pathway may contribute to plasticity or secretion. Voltage-gated Ca^{2+} influx was investigated using trains of action potentials delivered at 5 Hz, 10 s (mimicking the synaptic input that initiates the afterdischarge) or 5 Hz, 1 min (mimicking the fast phase of the afterdischarge). While both trains transiently elevated intracellular Ca^{2+}, only the 5 Hz, 1 min stimulus produced a Ca^{2+} rise that markedly outlasted the initial influx, consistent with Ca^{2+}-induced Ca^{2+} release (CICR). Thus, robust Ca^{2+} influx is required to trigger bag cell neuron CICR.

Depletion of the smooth endoplasmic reticulum store, with Ca^{2+}-ATPase blocker, cyclopiazonic acid, or collapse of the mitochondrial membrane potential, with

protonophore, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, significantly attenuated CICR. Furthermore, application of tetraphenylphosphonium, a blocker of mitochondrial Ca^{2+} release, also reduced CICR. Dual depletion of the endoplasmic reticulum and mitochondria did not reduce CICR further than depletion of mitochondria alone. Thus, in departure from the traditional mechanism of CICR, bag cell neuron CICR relies ultimately on mitochondrial Ca^{2+} release. Finally, intracellular Ca^{2+} changes during, and after, a stimulus mimicking the full afterdischarge were enhanced by PKC activation. Activity-dependent induction of CICR in bag cell neurons has the potential to influence secretion, membrane currents, or gene expression.

Contributions

The following thesis expands on preliminary discoveries made in our laboratory by Dr. Neil Magoski and Babak Kachoei (research student, MD) regarding both Ca^{2+} influx and intracellular Ca^{2+} stores in bag cell neurons. Dr. Magoski provided me with initial training in sharp- and whole-cell recording, as well as fluorescence imaging. Dr. Magoski and I discussed on a regular basis, results and observation, experimental procedures, and relevant literature.

I performed all of the data recordings and analysis contained in this thesis. Most of the solutions and drugs were prepared by myself. However, our laboratory technician, Ms. Shannon Smith, also provided assistance in some solution preparation. In addition, Ms. Smith provided animal care services. Although I performed all cell culture activities for the cells that I intended to use, animal dissections and removal of the abdominal ganglia were shared between myself and other members of the lab.

Special recognition should go to Dr. Steve Iscoe for providing use of his sharp-electrode puller in our laboratory. In addition *Conus textile* venom was provided thanks to Dr. B.M. Olivera and Ms. J.S. Imperial.

I prepared this thesis and all of its contents with necessary editing, proofreading, and constructive criticism from Dr. Magoski. This work will be summarized in two manuscripts, written together with Dr. Magoski. One paper will be based entirely on data presented here concerning Ca^{2+}-induced Ca^{2+} release, while the other paper will include the data on the Mn^{2+}-quench of fura along with single-channel work from Dr. Magoski.

Acknowledgements

I would like to thank Dr. Magoski for providing me with thorough and patient instruction, guidance, and support throughout my degree. Moreover, I would like to thank him for the opportunity to work in his laboratory and for encouraging me to consider scientific research in the first place. His generosity, dedication, and enthusiasm, cannot go unmentioned.

Throughout my degree I was fortunate enough to work alongside extraordinary lab mates. I would like to thank Ms. Shannon Smith (our laboratory technician), Ms. Anne Hung (MSc graduate), and Ms. Kate Gardam (MSc candidate) especially, for their unwavering support, consistent thoughtfulness, and endless humour. Also, I would like to acknowledge all of the other members of the Magoski lab, past and present, who together created an environment that was welcoming, fun, memorable, and also productive (for the most part).

In addition, I would like to thank the department of Physiology, Queen's University, CIHR, CFI, and OIT for supporting my research interests.

Lastly, I would like to dedicate this thesis to my parents, my brother, extended family, 302 Ladies, and all of my friends for their dedication, love, and support.

Table of contents

List of Figures

List of Abbreviations

α_{1C}	alpha 1 C subunit of calcium channel
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
Ba^{2+}	barium ion
cADP	cyclic adenosine diphosphate
Ca^{2+}	calcium ion
CaM	calmodulin
CaXΔ	calcium exchanger
cfASW	calcium free artificial sea water
CICR	calcium-induced calcium release
Cl^-	chloride ion
CPA	cyclopiazonic acid
CRAC	Ca^{2+} release activated Ca^{2+} channel
CtVm	*Conus textile* venom
d	day
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglion
EGTA	ethyleneglycol bis (aminoethylether) tetraacetic acid
ELH	egg-laying hormone
ER	endoplasmic reticulum
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
fS	femto siemens
G-protein	guanosine triphosphate binding protein
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethlpiperazine-N'-2-ethanesulphonic acid
h	hour
H^+	hydrogen ion
Hz	hertz (1/s)
IC_{50}	50% inhibitory concentration
IP_3	inositol 1,4,5-triphosphate
IP_3R	inositol 1,4,5-triphosphate receptor
K^+	potassium ion
kPa	kilo pascals
MΩ	megaohms
MCU	mitochondrial Ca^{2+} uniporter
Mg^{2+}	magnesium ion
MIT	mitochondria
Mn^{2+}	manganese ion
mRNA	messenger ribonucleic acid
ms	millisecond
mV	millivolt
n	sample number

NA	numerical aperture
Na^+	sodium ion
nA	nanoamperes
nASW	normal artificial salt water
NMDA	N-methyl- D-aspartic acid D-glucamine
pH	negative logarithm of hydrogen ion concentration
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
pS	pico siemens
ROI	region of interest
ROC	receptor-operated channel
RuR	ruthenium red
RyR	ryanodine receptor
s.e.m.	standard error of the mean
SERCA	sarcoplamic-endoplasmic reticulum Ca^{2+}-ATPases
τ	time constant
tcASW	tissue culture artificial salt water
TTP	tetraphenylphosphonium
UV	ultra violet
W	watt
V_{mit}	mitochondrial membrane potential
v/v	volume per volume
VGCC	voltage-gated calcium channel

Chapter 1: Introduction

Intracellular Ca^{2+} - a fundamental biochemical and physiological signal

Divalent ions, Ca^{2+} and Mg^{2+}, are ubiquitous in biological solutions, as they uphold crucial roles in many cellular functions (White & Hartzell, 1989). Although the maintenance of both Ca^{2+} and Mg^{2+} concentrations is critical, Ca^{2+} is more frequently and abundantly sequestered compared to Mg^{2+}. Whether due to influx from the extracellular environment or release from intracellular stores, Ca^{2+} initiates numerous processes through transferring an electrical charge or initiating a biochemical signal. Some physiological roles for Ca^{2+} include muscle contraction, gene expression, metabolism, secretion, neuronal signalling, and synaptic plasticity (Ringer, 1883; Hardingham *et al.* 1998; Pozzan *et al.* 1994; Duan *et al.* 2003; Borst & Sakmann, 1996; Kostyuk & Verkhratsky, 1994).

Despite similarities with voltage-gated Na^{+} and K^{+} channels, Ca^{2+} channels are unique in that they conduct an electrical charge that is often translated into a chemical signal (Hille, 2003). As such, Ca^{2+} serves as a second messenger that triggers intracellular responses. In addition, it is the only intracellular messenger that can enter directly by changes in membrane potential (through voltage-gated Ca^{2+} influx) and ligand binding (through Ca^{2+}-permeable ionotropic receptors or receptor-operated Ca^{2+} influx and release), while also acting as its own amplifier (Ca^{2+}-induced Ca^{2+} release) and providing a mechanism for negative feedback (Ca^{2+}-dependent inactivation of Ca^{2+} channels).

In neurons, there is typically a 10, 000-fold Ca^{2+} concentration gradient from outside to inside that allows for transient elevation in intracellular Ca^{2+} during action

potential firing (Hille, 2003; Levitan & Katzmarek, 2002). Previous studies using the squid giant synapse revealed that near-membrane Ca^{2+} microdomains reach concentrations above 100 μM during action potential firing (Llinas *et al.* 1992). Similarly, at the ribbon synapse of retinal bipolar cells, the location of the ready-releasable pool of vesicles spatially corresponds with submembranous Ca^{2+} microdomains (Beaumont *et al.* 2005). The transfer and release of vesicles from this pool requires at least 1 μM Ca^{2+}. To achieve these types of changes in concentration, intracellular Ca^{2+} must be tightly regulated via influx, sequestering, and extrusion mechanisms.

Voltage-gated Ca^{2+} influx

Ca^{2+} influx occurs primarily through voltage-gated Ca^{2+}channels, which were discovered inadvertently in *Crustacean* muscle fibres (Fatt & Katz, 1953). Small and transient Ca^{2+} signals, later to be called "calcium spikes", were unmasked when sodium ions were omitted from the medium. Subsequently, voltage-gated Ca^{2+} channels have been found in all excitable cells (Hagiwara, 1983).

Based on activation properties, Ca^{2+} currents are classified as low-voltage activated (Akaike, 1991; Huguenard, 1996) or high-voltage activated (Bean 1989; Carbone & Swandulla, 1989; Kostyuk, 1989; Tsien *et al*. 1988). Small and transient, T-type Ca^{2+} channels activate at potentials ~20-30 mV more negative than most high-voltage activated currents, and inactivate at holding potentials more positive than -50 mV (Huguenard, 1996). However, Ca^{2+} influx occurs primarily through high voltage-activated Ca^{2+} channels. These channels, of which there are numerous types with associated functions, are opened only when the membrane potential is sufficiently

depolarized, such as during an action potential (Hille, 2003; Ertel *et al*. 2000). For example, slow and long-lasting L-type Ca^{2+} channels are activated at holding potentials positive to -10 mV and often initiate peptide and hormonal release (Nowycky *et al*. 1985). However, there are exceptions such as low-voltage activated components in both bag cell neuron Ca^{2+} current (Tam & Magoski, unpublished), as well as low threshold L-type Ca^{2+} channels in dopaminergic substantia nigra neurons (Durante *et al.* 2004). R- and N- type Ca^{2+} channels, on the other hand, are fast inactivating and are involved mainly in the release of small molecule transmitters, such as amino acids. P/Q- type channels are larger and especially fast (Llinas *et al*. 1989; Zhang *et al*. 1993). The categorization of these Ca^{2+} channel types is based on their physical distribution, structural make-up, and activation/inactivation properties.

Early Ca^{2+} channel studies, conducted on *Aplysia*, revealed that unlike other voltage-gated ion channels, which inactivate exclusively via a voltage-dependent mechanism, Ca^{2+} channels are also inactivated by the entry of Ca^{2+} itself (Tillotson, 1979). This mode of Ca^{2+}-dependent inactivation has subsequently been shown to be a result of Ca^{2+} binding to calmodulin, a Ca^{2+}-sensing, channel-associated protein (Zuhlke *et al*. 1999). In addition to the channel, calmodulin associates with a number of proteins. Thus, as Ca^{2+} binds to calmodulin, it may modulate the function of proteins in near proximity (Nelson & Cox, 2000). Autoregulation of the channel depends on other factors; for example, the carboxy terminus of channel subunit α_{1C} (also known as $Ca_v1.2$) contains an isoleucine-glutamate motif, and just upstream is a consensus EF-hand motif. Both motifs appear to be involved in the inactivation of the channel, possibly fulfilling a Ca^{2+} transduction role (Peterson *et al*., 2000).

Receptor-operated Ca^{2+} influx and release

Although most Ca^{2+} influx is a result of voltage-gated channel activity, there are a number of Ca^{2+} permeable receptors which are not voltage-dependent, but contribute to Ca^{2+} entry. Receptor-operated influx occurs through ligand-gated ionotropic receptors or metabotropic receptors that indirectly trigger other plasma membrane or intracellular channels. The basis of both mechanisms relies on the binding of a neurotransmitter. In the case of Ca^{2+}-permeable ligand-gated ionotropic receptors, a transmitter binds to a receptor that is itself a nonselective cation channel (Purves *et al.*, 2001). Subsequently, Na^{+}, K^{+}, and Ca^{2+} ions can flow across the membrane. Some of the most common of these channels are N-methyl-D-aspartic acid (NMDA) and to a lesser extent Ca^{2+}-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. NMDA receptors are an exception in that they require, in addition to glutamate binding, sufficient depolarization to remove a Mg^{2+} ion which blocks the pore at resting potentials. In addition, Ca^{2+} influx can occur via nicotinic (Mulle *et al*., 1992), purinergic (Fine *et al*., 1989), and serotonergic (Nichols & Mollard, 1996) ionotropic receptors.

Metabotropic receptors promote Ca^{2+} entry through a process mediated via GTP-binding (G) proteins (Simon *et al*. 1991; Levitan & Kaczmarek 2002). When bound by a ligand, the receptor-linked trimeric G-protein dissociates, allowing the alpha subunit to interact with various target proteins, including membrane bound phospholipase C. Activated phospholipase C cleaves the membrane constituent phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 travels rapidly throughout the cytosol and subsequently binds to IP_3-gated Ca^{2+} release receptors on the

endoplasmic reticulum (ER), which results in a large Ca^{2+} release (see subsequent section). Diacylglycerol, although implicated in other signalling roles, activates protein kinase C (PKC), which can phosphorylate a variety of target proteins. In rat dorsal root ganglion (DRG) neurons for example, PKC accelerates Ca^{2+} uptake into the ER (Usachev *et al*. 2006). Collectively, G-proteins, second messengers, or enzymes can also promote Ca^{2+} influx by activating Ca^{2+} permeable ion channels at the plasma membrane (Plant & Schaefer, 2003).

The ER is the principal intracellular Ca^{2+} source, creating a vast network of tubules throughout the neuron and maintaining a 1000-fold concentration gradient compared to the cytosol (Bardo *et al.* 2005a,b). This gradient is the result of several Ca^{2+} pumps and exchangers. In addition, passive channels in the ER membrane, called presenilins, play a major role in Ca^{2+} leak from the ER (Tu *et al*. 2006). When compared to buffers in the cytosol, the buffers in the ER lumen have a relatively low affinity for Ca^{2+} (Verhkhratsky, 2005). This ensures that Ca^{2+} can be released rapidly when stimulated to do so. As well as providing a source for rapid Ca^{2+} release, the ER serves to protect neurons against neurodegeneration when the Ca^{2+} levels are dangerously high (Franklin *et al*. 2001).

To counterbalance Ca^{2+} efflux from the ER, sarcoplamic-endoplasmic reticulum Ca^{2+}-ATPases (SERCA) continuously pump Ca^{2+} into the ER. The activity of SERCA is regulated by chaperone proteins from both sides of the ER membrane. Calreticulin on the luminal side, interacts with the pump directly, activating SERCA once Ca^{2+} in the ER drops (Baksh & Michalak, 1991). The transmembrane protein, calnexin, acts as a Ca^{2+} switch from the cytosolic surface (Rodericka *et al.* 2000). High intracellular Ca^{2+}

concentrations cause dephosphorylation of calnexin, and its subsequent dissociation from SERCA activates the pump.

Intracellular Ca^{2+} release channels

Ca^{2+} efflux from the ER is controlled by two families of intracellular ionotropic-like Ca^{2+} channels: IP_3 receptors (IP_3Rs), which are activated by IP_3 and inhibited by Xestospongin C (Gafni *et al*. 1997), and ryanodine receptors (RyRs), which are blocked by the plant alkaloid, ryanodine, and activated by Ca^{2+} itself (Meissner, 1986). Although both of these receptor types perform a similar function, they differ greatly in their regulation (Meldoesi, 2001).

IP_3Rs are controlled by several regulators, including serine-threonine kinases, tyrosine kinases, Ca^{2+}-dependent phosphatase, and calcineurin (Meldolesi, 2001). Stimulation of either RyRs or IP_3Rs can cause rapid Ca^{2+} release. Since IP_3Rs and RyRs are sensitive to both intracellular second messengers and Ca^{2+}, these channels respond to signals originating at the plasma membrane and to cytosolic Ca^{2+} (Melsolesi, 2001). It is possible that Ca^{2+}channels in the plasma and ER membranes are coupled directly, as indicated by coimmunoprecipitation of RyRs and certain Ca^{2+} channel subunits (Ouardouz *et al.* 2003).

Less is known about RyRs in neurons, because for years they had only been observed in striated muscle (Meissner, 1986; Imagawa *et al*. 1987). More recently, three RyR types have been found in the mammalian CNS, with the expression of type 2 being the most prominent (Verkhratsky, 2005). Regulation of RyRs depends both on the concentration of Ca^{2+} in the ER lumen and in the cytosol. Additional endogenous modulators of RyRs include Mg^{2+} (Murayama *et al.* 2000), calmodulin (Rodney *et al.*

2005), calexcitin (Nelson *et al.* 1999), and as demonstrated first in *Aplysia*, cADP-ribose (Vu *et al*. 1996; Thomas *et al.* 2001).

Ca^{2+}-induced Ca^{2+} release

Ca^{2+} elevations in the cytosol may trigger the release of additional Ca^{2+} from the ER, via Ca^{2+} opening RyRs, in a process known as Ca^{2+}-induced Ca^{2+} release (CICR). This amplifying phenomenon was first discovered in myocytes (Endo *et al*. 1970; Fabiato & Fabiato, 1975), and subsequently found in neurons of *Aplysia* (Gorman & Thomas, 1980), bullfrog (Smith *et al*., 1983; Lipscombe *et al*, 1988), and rat (Neering & McBurney, 1984). It appears to have a threshold for concentration and rate of rise of Ca^{2+}, meaning too small a Ca^{2+} influx is not enough to stimulate CICR (Verkhratsky, 2005). Once the threshold is met, however, in some neurons, such as those in the DRG of rats, an all-or-none response is observed (Usachev & Thayer, 1997; Cohen *et al.* 1997). In other neurons, CICR is evoked in a graded fashion, whereby one action potential or a single depolarization stimulates CICR, and an increase in the number of action potentials/depolarizations augments CICR (Gorman & Thomas, 1980; Hua *et al*. 1993; Orkand & Thomas, 1995). Finally, initiation of some neuronal CICR requires a true burst of action potentials and a prominent Ca^{2+} influx (Smith *et al.* 1983; Neering & McBurney, 1984; Schmigol *et al*. 1995; Richter *et al*. 2005).

Typically, CICR is due to voltage-gated Ca^{2+} influx in turn activating RyRs (Fig. 1). Not surprisingly, almost all neurons are sensitive to micromolar concentrations of the blocker, ryanodine (Weber, 1968; Imagawa *et al*, 1987). In addition, CICR is inhibited by micromolar concentrations of cyclopiazonic acid (CPA; Seidler *et al*. 1989), a SERCA

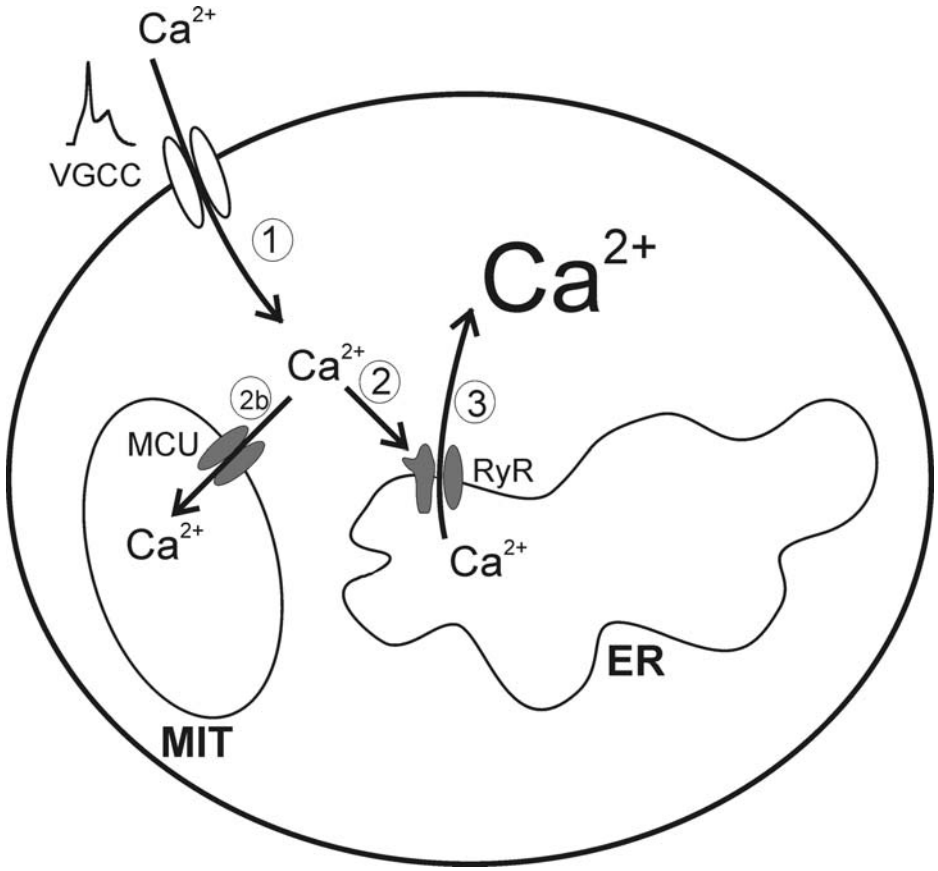

Figure 1. **Traditional Ca^{2+}-induced Ca^{2+} release (CICR)**

A schematic showing the regulation of intracellular Ca^{2+} and initiation of CICR. Ca^{2+} entry is regulated by voltage gated Ca^{2+} channels (VGCC; step 1). A robust increase in Ca^{2+} stimulates ryanodine receptors (RyRs; step 2) present on the ER membrane to release stored Ca^{2+} in a process known as CICR (step 3). Upon entry, Ca^{2+} is also buffered by mitochondria via the mitochondrial uniporter (MCU; step 2a).

inhibitor that depletes the ER of Ca^{2+}, or millimolar concentrations of caffeine, a RyR agonist which also depletes the ER of Ca^{2+} (Rousseau *et al*., 1988).

Other intracellular Ca^{2+} stores

Other organelles, besides the ER, handle intracellular Ca^{2+}. Acidic stores, such as lysosomes, appear to maintain significantly higher concentrations of Ca^{2+} compared to the cytosol (Yagodin *et al.* 1999). Mitochondria also serve as intracellular Ca^{2+} stores and thereby propagate Ca^{2+} signals. Previous work has demonstrated that mitochondrial Ca^{2+} is involved in post-tetanic potentiation (Tang & Zucker, 1997; Zhong *et al*. 2001), exocytosis (Giovannucci *et al.* 1999; Billups & Forsythe, 2002), and the modulation of CICR (Friel & Tsien, 1994; Jackson & Thayer, 2006).

The Ca^{2+} concentration within mitochondria is typically in the micromolar range (Miyata *et al*. 1991), while the mitochondrial membrane potential (V_{mit}) is very negative with respect to the cytosol, roughly -180 mV (Johnson *et al*. 1981; Ylitalo *et al*. 2000). The reversal potential for Ca^{2+} in the mitochondria is much more positive than V_{mit}; consequently, the driving force for Ca^{2+} is into the mitochondria. This promotes uptake by the mitochondria via an ion channel known as the mitochondrial Ca^{2+} uniporter (Kirichok *et al.* 2004). Conversely, Ca^{2+} exits the mitochondria if V_{mit} collapses, as this would render the reversal potential more negative than V_{mit}. Mitochondrial Ca^{2+} release occurs physiologically via Na^{+}/Ca^{2+} and H^{+}/Ca^{2+} exchangers (Pauceka & Jabůrekb, 2004).

Because mitochondria are often located near Ca^{2+} release channels on the ER or Ca^{2+} influx channels on the plasma membrane, mitochondria can sequester Ca^{2+} as it enters the cytosol both from the extracellular environment or the ER (Colegrove *et al.*

2000a,b; see Fig. 1). Mitochondria thus buffer Ca^{2+} from the cytoplasm during influx, especially at the synaptic terminal (Kim *et al*. 2005). Sequestering Ca^{2+} in this manner may limit Ca^{2+}-dependent inactivation of Ca^{2+} channels or stimulation of Ca^{2+}-activated K^+ channels (Malli *et al.* 2003). After sequestering Ca^{2+}, mitochondria may release it slowly back into the cytosol, exchanging it between the ER and cytosolic binding proteins, thus potentiating local Ca^{2+} buffering and ER refilling (Arnaudeau *et al*. 2001). Also, this trans-mitochondrial Ca^{2+} flux could strengthen the activation of store-operated Ca^{2+} channels (see below), possibly enhancing CICR (Marhl *et al.* 2000).

Store-operated Ca^{2+} influx

Store-operated Ca^{2+} influx is a mechanism of Ca^{2+} entry, whereby depletion of intracellular Ca^{2+} stores activates specific Ca^{2+}-permeable channels in the plasma membrane (Casteels & Droogmans, 1981; Nilius, 2003; Parekh, 2003). Such channels are categorized as either Ca^{2+} release activated Ca^{2+} (CRAC) channels or non-selective cation channels. CRAC channels exhibit a very small conductance (~20 fS; Hoth & Penner, 1993; Zweifach & Lewis, 1993; Prakriya & Lewis, 2003), while the conductance of store-operated non-selective cation channels is significantly higher (tens of pS; Su *et al*. 2001; Curtis & Scholfield, 2001; Trepakova *et al*. 2001; Albert & Large, 2002).

Initially, store-operated Ca^{2+} influx was thought to exist only in non-neuronal cells (Casteels & Droogman, 1981). Neurons were thought to lack store-operated Ca^{2+} influx (Jackson *et al*. 1988; Stauderman & Pruss, 1989; Friel & Tsien, 1992) until a discovery by Clementi *et al*. (1992) in a pheochromocytoma cell line from rat. Since then, store-operated Ca^{2+} influx has been described in various neurons and neuroendocrine cells (Villalobos & Garcia-Sancho, 1995; Powis *et al*. 1996; Piper &

Lucero, 1999; Liu & Gylfe, 1997; Bouron, 2000; Prothero *et al*. 2000), including a report from our laboratory on the bag cell neurons of *Aplysia* (Kachoei *et al*. 2006).

The bag cell neurons of *Aplysia californica*

The marine mollusc, *Aplysia californica*, has proven useful in studying the neural control of behaviour, pattern-generation, learning, neuroendocrine function, and long-lasting changes in excitability (Kandel, 1976; Byrne and Kandel 1996). With several identifiable neurons controlling distinct behaviours, many nerve cells from *Aplysia* have been characterized, including the bag cell neurons. Located within the abdominal ganglia, at the base of the pleuroabdominal nerves, the bag cell neurons form two clusters comprised of 200-400 neurons each (Fig. 2; Kupfermann, 1967; Kupfermann & Kandel, 1970). These neurons are numerous, absolutely identifiable, amenable to electrophysiology (diameter 20-60 μm), and consistently respond to environmental stimuli by initiating characteristic behaviours associated with reproduction (Pinsker & Dudek, 1977; Dudek *et al*., 1979). The bag cell neurons have been used extensively to examine aspects of ion channel regulation, peptide release, the neural control of behaviour, and, of particular interest to the present study, intracellular Ca^{2+} regulation.

Following a brief synaptic input, these neurons undergo a 30-minute period of repetitive firing, known as the afterdischarge (Fig. 3; Kupfermann, 1967; Conn & Kaczmarek, 1989). Concomitant with the afterdischarge, intracellular Ca^{2+} levels increase rapidly, triggering the release of several neuropeptides which initiate egg-laying behaviour ~30 min following the termination of the afterdischarge (Arch, 1972a,b; Stuart *et al*., 1980; Loechner *et al*., 1990). The neuropeptides include a variety of so-called bag cell peptides and most importantly, egg laying hormone (ELH; Chiu *et al*., 1979; Scheller

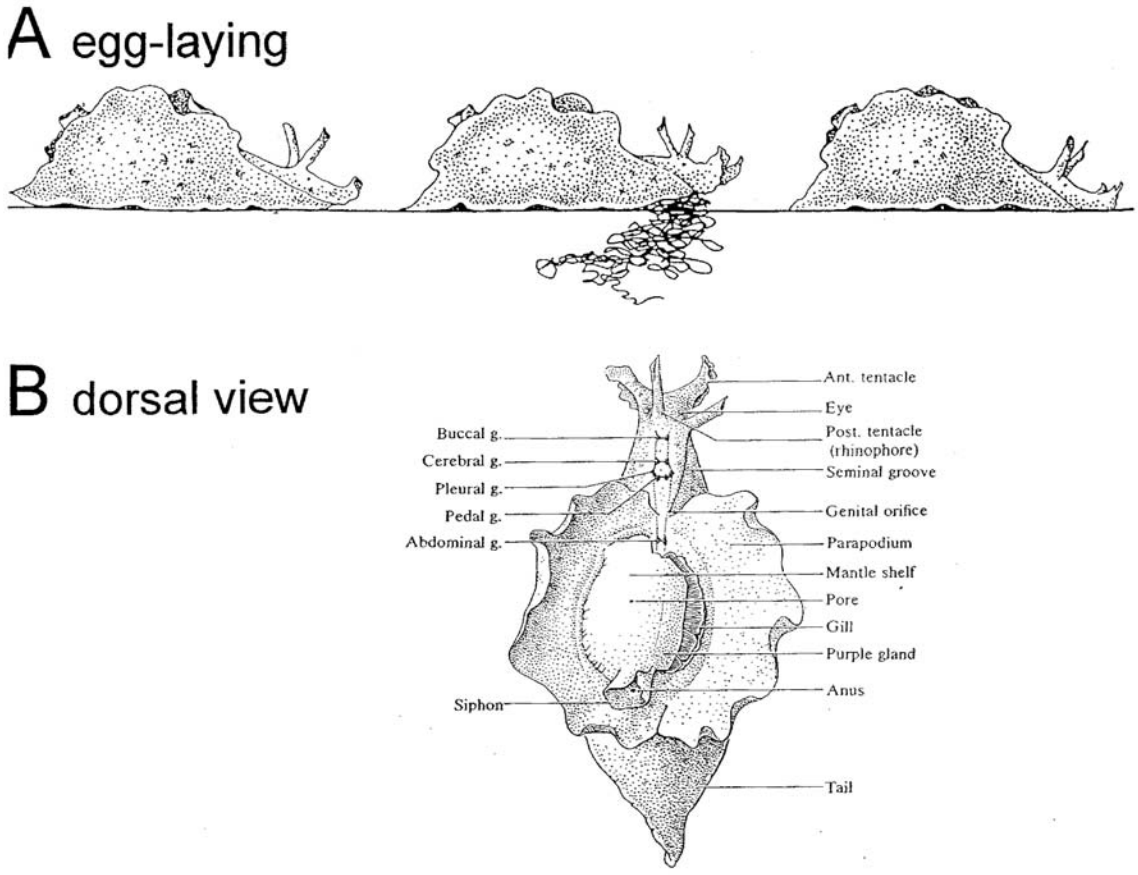

Figure 2. **Control of egg-laying behaviour in *Aplysia californica***
A, Egg-laying in *Aplysia*. The egg mass is the string-like structure near the head of the animal, appearing in the middle panel.
B, A dorsal view of *Aplysia* identifying major organs and the central nervous system. Note the location of head and abdominal ganglia. Both A and B are modified from Kandel (1976).

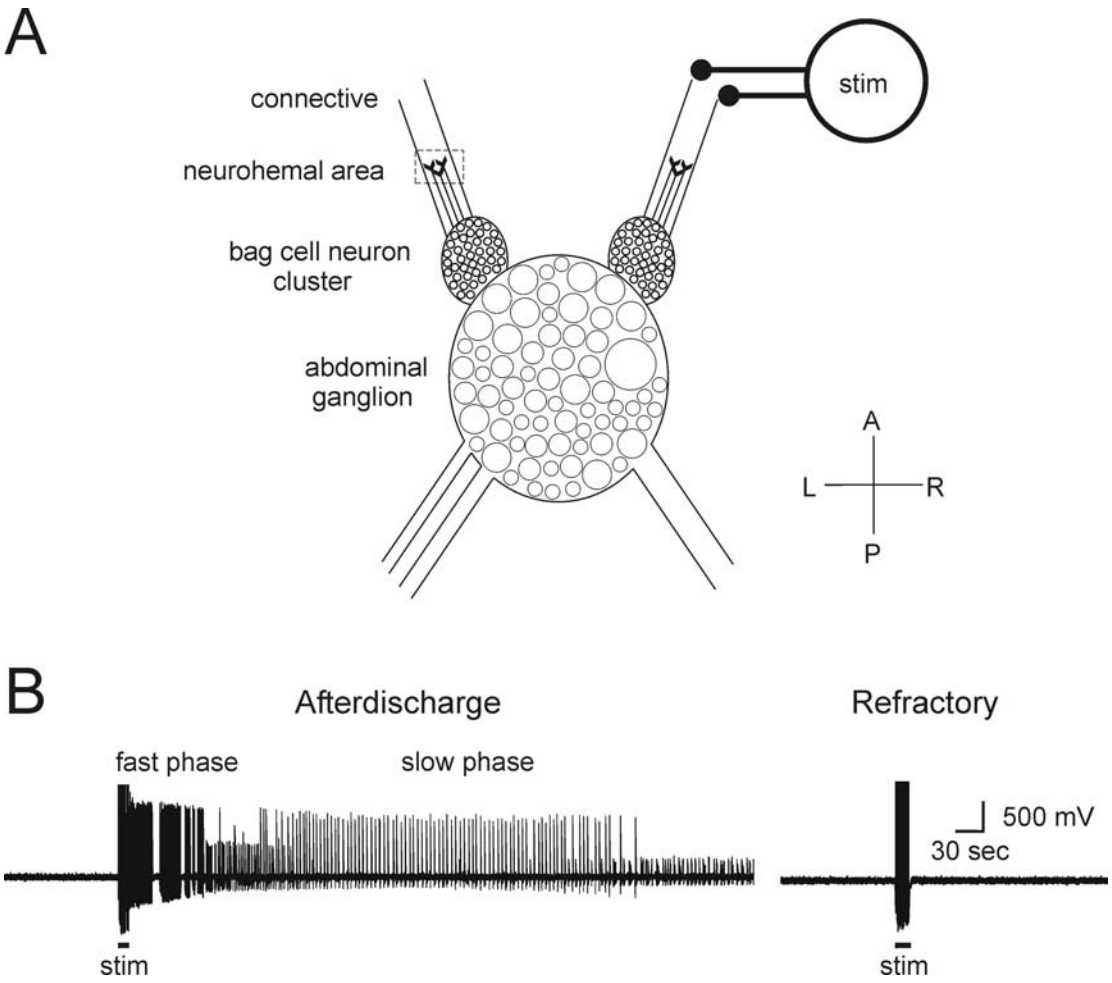

Figure 3. **The bag cell neuron afterdischarge and refractory period**
A, A schematic of the abdominal ganglia and the two bag cell neuron clusters. The afterdischarge (see panel B) is induced by a brief stimulus of synaptic afferents in the pleural connective which leads to one of the bag cell neuron clusters. Courtesy of N.S. Magoski.
B, *Left*, An extracellular recording of bag cell neurons in the intact cluster during the afterdischarge, which consists of both a fast and a slow phase of action potential firing (record truncated for display).
The afterdischarge can last up to 30 min, during which ELH is released from the neurohemal area into circulation. *Right*, An extracellular recording from a refractory cluster. Subsequent to the termination of the afterdischarge, bag cell neurons are refractory for ~18 h. During this period additional stimuli are unable to elicit a second afterdischarge. Modified from Magoski & Kaczmarek (2005).

et al., 1983). The latter acts on both central neurons and peripheral targets, principally the ovitestis, to produce a series of behaviours that culminate in egg mass deposition (Mayeri *et al*. 1979a,b; Stuart & Strumwasser, 1980; Rothman *et al*., 1983). The duration of the afterdischarge is independent of the number of stimuli, so that once initiated, it will run a full course (an all-or-none response). This pattern of firing is similar to the bursting observed in other peptide-secreting cells. For instance, the firing pattern of oxytocin secreting neurons in the hypothalamus is strikingly similar to the afterdischarge (Armstrong *et al.* 1994). In that case, however, the high frequency bursts stimulates the release of oxytocin resulting in milk ejection. Similarly, insulin secretion from pancreatic β-cells is elicited by rapid action potential firing in response to elevated glucose (Ashcroft & Rorsman, 1989).

Bag cell neurons are electrically coupled within a cluster and to the cells in the contralateral cluster, thus, they serve as pacemakers for one another (Kupfermann & Kandel, 1970; Blankenship & Haskins, 1979). When stimulated, the pattern consists of a short phase of rapid firing followed by a prolonged phase of slow firing. During the first phase, voltage-dependent K^+ channels are inhibited and at least two species of cation channel are activated to depolarize the membrane to ~ -30 mV from a resting potential of -60 mV (Kaczmarek & Strumwasser, 1984; Wilson *et al.* 1996; Whim & Kaczmarek, 1998; Hung & Magoski, 2007). This results in rapid firing (~1 min at 2-6 Hz) with action potentials reaching +20 to +50 mV at their peak. The second phase of slow firing (~30 min at 0.5-1 Hz) coincides with spike broadening and an increase in voltage-gated Ca^{2+} current (Strong *et al.* 1987). The early phase is dependent on Na^+ influx. This was demonstrated when stimulated bag cells failed to show a fast phase in Na^+-free saline

(Kaczmarek *et al.* 1982). The characteristic firing pattern of the second phase was unaltered, as it appears to be Ca^{2+} dependent. When stimulated in Ca^{2+}-free media, afterdischarges show high firing rates exclusively, i.e. fast phase only. Under normal circumstances, Na^{+} and Ca^{2+} flow through cation channels, as mentioned above, to provide the depolarizing drive for both phases of the afterdischarge (Wilson *et al.* 1996). Parenthetically, the voltage-gated Ca^{2+} current (see below) mediates the upstroke of the action potential in bag cell neurons much like a Na^{+} current would.

As mentioned previously, conventional PKC is activated by diacylgylcerol and Ca^{2+} binding. During the second phase of the afterdischarge, both Ca^{2+}-dependent and -independent *Aplysia* PKCs are activated (Wayne *et al.* 1999). PKC can modulate the function of ion channels. The first report of this phenomenon was in the bag cell neurons themselves, and indicated that PKC activation enhances Ca^{2+} current during the second phase of the afterdischarge (DeRiemer *et al*. 1985; Conn *et al*. 1987). The current hypothesis regarding enhanced Ca^{2+} current suggests that PKC causes the insertion of a second, larger conductance Ca^{2+} channel into the plasma membrane to accompany the always present, smaller conductance Ca^{2+} channel (Strong *et al*. 1987). As two pools of Ca^{2+} channels are reported in bag cell neurons, one primarily located at the membrane and another primarily in vesicles, it is probable that vesicular channels are PKC sensitive, while membrane channels are responsible for basal Ca^{2+} current (White & Kaczmarek, 1997).

An 18 h refractory period follows the afterdischarge. During this state, another prolonged afterdischarge cannot be evoked, no matter the frequency and/or duration of the stimulus, although the neurons are excitable (Kupferman & Kandel, 1970; Kaczmarek

et al. 1982). The mechanisms responsible for the onset of the refractory period are not fully understood, although it has been suggested that Ca^{2+} entry is important for its initiation (Kaczmarek & Kauer, 1983). Thus, it appears that the Ca^{2+} influx stimulates both the transition from the fast to slow phase and events triggering the refractory period. For example, when stimulated in low Ca^{2+} media, bag cell neurons failed to exhibit refractoriness (Kaczmarek *et al.* 1982). Also, Magoski *et al*. (2000) provided indirect evidence that Ca^{2+} influx through one of the cation channels discussed earlier is important for rendering the neurons refractory. *One goal of this thesis is to address whether the Ca^{2+} influx through the cation channel actually occurs.*

The normal resting concentration of intracellular Ca^{2+} ranges between 150-300 nM in bag cell neurons (Fisher *et al*. 1994; Knox *et al*. 1996; Jonas *et al*. 1997; Magoski *et al*. 2000). During action potential firing, however, the concentration increases significantly. Previous studies have demonstrated that *cytosolic* Ca^{2+} increases from ~300 nM to ~1 μM during the afterdischarge (Fisher *et al*. 1994; Michel & Wayne, 2002) or in response to agonist application (Kachoei *et al.* 2004). It is also clear that intracellular stores contribute to Ca^{2+} elevation during electrical activity in bag cell neurons (Fisher *et al.* 1994; Wayne *et al.* 1998a). For example, the Ca^{2+} liberating second messenger, IP_3, is elevated during the afterdischarge (Fink *et al*. 1988). As for Ca^{2+}, its levels follow a biphasic pattern (Fisher *et al.* 1994). Immediately following afferent stimulation, there is a rapid rise in cytosolic Ca^{2+}, which declines just as rapidly. Yet a second slower Ca^{2+} elevation follows, which often outlasts the duration of the stimulus. The second elevation, responsible for this "Ca^{2+} shoulder", may be due to Ca^{2+} released from intracellular stores or the consequence of delayed uptake and extrusion of

Ca^{2+}.

It has yet to be determined whether the prolonged elevation seen in bag cell neurons during the afterdischarge is a result of CICR or a combination of mechanisms acting in concert upon Ca^{2+} entry. Moreover, there is limited information concerning the Ca^{2+} changes under firing patterns which correspond to stimuli that neurons would experience during the synaptic input which evokes the afterdischarge or the fast and slow phases of the burst itself. Using a Ca^{2+} sensitive electrode, Fisher *et al*. (1994) showed that Ba^{2+} (substituted for Ca^{2+}) entry into bag cell neurons caused a subsequent, prolonged release of Ca^{2+}. This suggests that CICR is possible in bag cell neurons. Such substantial changes in intracellular Ca^{2+} which accompany or even outlast depolarization could initiate secretion or the transcription of genes pertinent to reproduction, as well as activate large-conductance Ca^{2+}-activated K^{+} channels and/or cation channels, both of which are tied to the initiation of the refractory period (Magoski et al. 2000; Zhang *et al*. 2002; Lupinsky & Magoski, 2006). *The second goal of this thesis is to determine if CICR can occur in bag cell neurons and what Ca^{2+} changes take place during various action potential firing patterns.*

Hypothesis and Significance

This thesis examines Ca^{2+} changes that occur during and after pharmacological or electrical activation of cultured bag cell neurons. I will use simultaneous dye-imaging and whole-cell voltage-clamp or sharp-electrode current-clamp to test the following hypotheses:

1) Receptor-mediated activation of a non-selective cation channel results in Ca^{2+} influx.

2) Activity-dependent elevation of Ca^{2+} produces a subsequent release of Ca^{2+} from an intracellular store, i.e. CICR.

Because Ca^{2+} entry through the cation channel in question has been implicated in giving rise to the refractory period (Magoski *et al*. 2000), it is important to determine if indeed it allows Ca^{2+} into bag cell neurons. Regarding CICR, confirming this phenomenon in bag cell neurons would be consistent with the concept of Ca^{2+} as a signal to initiate change in neuronal function, and may well provide new mechanistic information on how neurons translate short-term activity into long-term signals.

Chapter 2: Materials and Methods

Animals and cell culture

Primary cultures of isolated bag cell neurons were obtained from adult *Aplysia californica* weighing 150-450 g. Animals were obtained from Marinus (Long Beach, CA, USA) and housed in an ~300 l aquarium containing continuously circulating, aerated artificial seawater (Instant Ocean; Aquarian System Inc.; Mentor, OH, USA) at 14-16°C on 12/12 h light/dark cycle and fed Romaine lettuce 3-5x/week. Following anaesthesia by injection of isotonic $MgCl_2$ (~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 $CaCl_2$, 55 $MgCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.8 supplemented with glucose (1 mg/ml), penicillin (100 units/ml), and streptomycin (0.1 mg/ml). The ganglia were 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 regular 35 x 10 mm polystyrene tissue culture dishes (430165; Corning, Corning, NY, USA). Cultures were maintained in tcASW for 1-3 d in a 14°C incubator. Experiments were carried out at 22ºC in normal ASW (nASW; composition same as tcASW but with glucose and antibiotics omitted). Salts were obtained from Fisher Scientific (Ottawa, ON, Canada) or Sigma-Aldrich (St. Louis, MO, USA).

Sharp-electrode current-clamp recording

Current-clamp recordings were made using an Axoclamp 2B amplifier (Axon

Instruments/ Molecular Devices; Sunnyvale, CA, USA) in bridge mode and the sharp-electrode method. Microelectrodes were pulled from 1.2 mm internal diameter, boroscilicate glass capillaries (IB120F-4; World Precision Instruments, Sarasota, FL, USA), with 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 using an IBM-compatible personal computer, a Digidata 1300 analogue-to-digital converter (Axon Instruments) and the Clampex acquisition program of pCLAMP 8.1 (Axon Instruments). Stimulation current was delivered using a S88 stimulator (Grass; Warwick, MA, USA). Electrophysiology was typically carried out simultaneously with Ca^{2+} imaging.

Whole-cell voltage-clamp

For some experiments, dye-filled neurons (see below) were whole-cell voltage-clamped at -60 mV using the Axoclamp and continuous voltage-clamp mode. Whole-cell microelectrodes (1.5 mm internal diameter, borosilicate glass capillaries, TW 150 F-4; World Precision Instruments) were fire polished to a resistance of 1.5-3.0 MΩ when filled with artificial intracellular saline (composition in mM: 500 K-aspartate, 70 KCl, 1.2 $MgCl_2$, 10 HEPES, 11 glucose, 5 EGTA, 10 reduced glutathione, 1 mM fura-PE3 (K^+ salt); 0110, Teflabs, Austin, TX, USA; Vorndran *et al*. 1995; pH 7.3 with KOH; free Ca^{2+} concentration set to 300 nM). The Ca^{2+} concentrations were calculated using the WebMaxC program. (http://www.stanford.edu/~cpatton/webmaxc/webmaxcE.htm). Simultaneous voltage-clamp and imaging was performed in ASW with the $CaCl_2$ equimolar replaced by $MnCl_2$. Under these conditions, the Mn^{2+} acts as a surrogate for

Ca^{2+}, whereby any Mn^{2+} entry through Ca^{2+}-permeable channels is detected by quenching of the fura (see results).

Ca^{2+} imaging

The Ca^{2+}-sensitive dye, fura-PE3, was pressure injected via sharp-electrode using a PMI-100 pressure microinjector (Dagan, Minneapolis, MN, USA), while simultaneously monitoring membrane potential with the Axoclamp (Fig. 4). 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 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 subsequent 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 30 min. 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 either 340 and 380 nm or 358 nm alone. 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

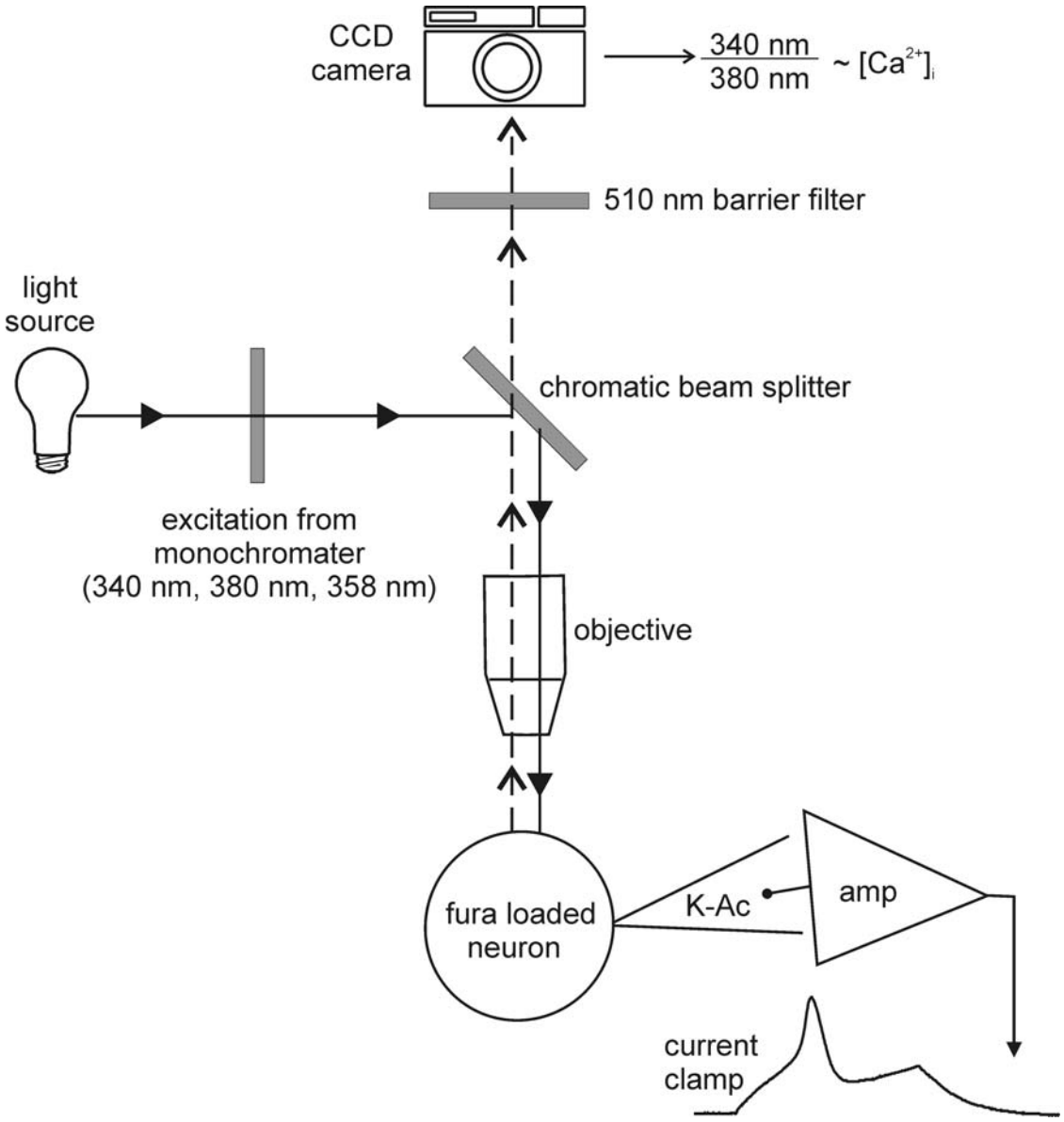

Figure 4. **Simultaneous fura imaging and sharp electrode recording**
A schematic representation of fluorescence imaging performed using a inverted microscope. Light from a Xenon arc lamp (light source) passes through a monochromatic illuminator which transmits light at wavelengths 340 nm, 380 nm, or 358 nm and excites the Ca^{2+} indicator, fura-PE3 within microinjected bag cell neurons. The emitted light then passes through a 510/40 nm barrier filter and is detected and intensified by a charge coupled device camera. Trains of action potentials are delivered via sharp electrode, while the fluorescence signal is recorded simultaneously.

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, there was a 50:50 distribution of blue and black pixels on the image display with no light going to the camera. Fluorescence intensities were sampled at 10, 20, or 60 s intervals using regions of interests (ROIs) defined over the neuronal somata prior to the start of the experiment and, if necessary, averaged 4-8 frames per acquisition. The emission following 340 and 380 nm excitation was ratioed (340/380) to reflect free intracellular Ca^{2+}, whereas emission following 358 nm excitation was recorded as an indicator of fura PE3 quench by Mn^{2+}. The black level determination, image acquisition, frame averaging, emitted light ROI sampling, and ratio calculations were carried out using the ImageMaster Pro software. Ratios or raw emission data were saved for subsequent analysis.

Measuring intracellular Ca^{2+}

The assessment of Ca^{2+} influx is experimentally difficult, and has been limited by the methods for measuring cytosolic free Ca^{2+}. Determining intracellular Ca^{2+} is problematic because potential techniques must be both selective and respond quickly to changes. Currently, the use of microscopy and fluorescent indicators are preferred. Fluorescent indicators emit altered fluorescence when bound to Ca^{2+}. Fura-2, an indicator used extensively in previous years (Grynkiewicz *et al*., 1985), has provided the backbone for more recent Ca^{2+} indicators. Fura-PE3 was developed because fura-2 had a tendency to leak out of cells and/or accumulate in organelles (Vorndran *et al.* 1995).

This newer indicator resists compartmentalization and leakage; for example, fura-PE3 remains in the cytosol because of added positive charge, and its slightly lower affinity for Ca^{2+} (K_d=204-290 nM) may render it more accurate than fura-2. That said, both fura 2 and fura-PE3 have a high affinity for Ca^{2+} (dissociation constant, K_d=161-224), and saturate in the low to mid micromolar range (Vorndran *et al.* 1995). Thus, fura-2 and fura-PE3 may under-report larger changes in intracellular Ca^{2+}.

Reagents and drug application

Drug application or solution exchanges were accomplished by manual perfusion using a plastic transfer pipette to exchange the bath (tissue culture dish) solution. In most cases, drugs were introduced directly into the bath by pipetting a small volume (<10 µl) of concentrated stock solution or a larger volume of saline (~500 µl) that was initially removed from the bath, mixed with the stock solution, and then reintroduced. Care was taken to perform all pipetting near the side of the dish and as far away as possible from the neurons. Cyclopiazonic acid (CPA; C1530; Sigma-Aldrich or 239805; Calbiochem, San Diego, CA, USA), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 21857; Sigma-Aldrich, ryanodine (559276; Calbiochem), phorbol 12-myristate 13-acetate (PMA; P8139; Sigma-Aldrich) 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 intracellular Ca^{2+}. Agents made up in water included ruthenium red (557450; Calbiochem) and tetraphenylphosphonium chloride (TTP; 218790; Sigma-Aldrich), which were dissolved as a stock in 500 µl water. Pre-treatment duration was 20-30 min, unless stated otherwise.

Conus textile venom (CtVm) (Cruz *et al*., 1976) lyophilate was provided by Dr. B.M. Olivera of the University of Utah (Salt Lake City, UT, USA). Crude CtVm was extracted using 0.5 % (v/v) trifluoroacetic acid (BP-618; Fisher-Scientific). Aliquots of CtVm were diluted into 2 ml of bath solution for a final protein concentration of ~ 100 μg/ml.

Analysis

Origin (version 7; OriginLab Corporation, Northampton, MA, USA) was used to import and plot ImageMaster Pro files as line graphs. For the Mn^{2+} quench experiments, the emission signal was fitted with a standard exponential using Origin. For intracellular Ca^{2+}, analysis used steady-state changes of the 340/380 ratio acquired by taking the average value, by eye or with adjacent-averaging, from regions that had reached steady-state. Time course of the recovery of Ca^{2+} signal was investigated at 50%, 75%, 90 % of baseline Ca^{2+}. Only time to 75% baseline is reported here, because in many cases Ca^{2+} levels did not return to 90% baseline, and differences where not always apparent at 50% recovery. In some cases, action potential amplitude and half-widths were determined using Clampfit (Axon Instruments) by setting cursors at the start and end of the spike.

Summary data are presented as the mean ± stardard error of the mean (s.e.m.). Statistics were performed on either raw 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 (with the Welch correction as required) t-test was used to test for differences between two means, while a standard one-way analysis of variance (ANOVA) with Dunnett's post-hoc test were used to test for differences between multiple means. If the

data were not normally distributed, a Mann-Whitney test was used to test for differences between two means, while a Kruskal-Wallis ANOVA and Dunn's post-hoc test were used to test for differences between multiple means. In some instances, a single mean was tested against zero using a one-sample t-test. Data were considered significantly different if the two-tailed p-value was <0.05.

Chapter 3: Results

Receptor-operated Ca^{2+} influx in bag cell neurons

One of the non-selective cation channels that drives the afterdischarge was suggested by Magoski *et al*. (2000) as a source of Ca^{2+} entry that may trigger the refractory period. A pharmacological agent that activates this channel, through what appears to be an intracellular pathway (Wilson & Kaczmarek, 1993; Magoski *et al*. 2002), is the venom (CtVm) from another marine snail, *Conus textile*. However, in addition to activating the channel, CtVm also releases Ca^{2+} from intracellular stores (Magoski *et al*. 2000). Thus, in determining if the CtVm-activated cation channel was Ca^{2+} permeable, it was important to distinguish between Ca^{2+} influx and Ca^{2+} release. To do so, fura-PE3-loaded cultured bag cell neurons were imaged at 358 nm while voltage-clamped at -60 mV. This wavelength respresents the isosbestic point of fura, i.e. where the fluoresence intensity is not sensitive to Ca^{2+}, but only determined by the concentration of the dye (Grynkiewicz *et al*. 1985). Replacing Ca^{2+} with Mn^{2+} in the extracellular medium allows for Mn^{2+} to act as a surrogate for Ca^{2+} and flow through any Ca^{2+} -permeable channels. When fura binds Mn^{2+} it quenches the dye and effectively reduces its concentration (Hallam & Rink, 1985; Sage *et al*. 1989). Thus, if CtVm opens a Ca^{2+} permeable cation channel, as well as causes intracellular Ca^{2+} release, only the Ca^{2+} (i.e. Mn^{2+}) entry will be detected. Moreover, keeping the voltage at -60 mV prevents the CtVm-activated channel from depolarizing the membrane potential and activating voltage-gated Ca^{2+} channels that would confound the assay.

To test that Mn^{2+} influx could indeed quench fura fluorescence in bag cell neurons, a positive control was performed by depolarizing the membrane potential for a

prolonged period to intentionally activate voltage-gated Ca^{2+} channels. In Mn^{2+} ASW, a 10 s step depolarization from -60 mV to +10 mV under voltage-clamp induced pronounced Mn^{2+} influx as measured by the rapid quench of fura-PE3 (Fig. 5). For the actual experiment, CtVm (100 μg/ml) was applied to neurons voltage-clamped throughout at -60 mV. In comparison to control, this produced a prominent inward current consistent with activation of the non-selective cation channel as reported by others (Fig 6A; Wilson *et al*. 1996). Moreover, CtVm application resulted in significant Mn^{2+} influx, presumably via the cation channel (Fig. 6*A*,*B*). Delivery of water, by comparison, resulted in little change as demonstrated by a significantly greater time constant. These results suggest that a receptor-operated Ca^{2+} influx pathway exists in bag cell neurons.

Changes to bag cell neuron Ca^{2+} following a brief train of action potentials

Short and long-term changes to intracellular Ca^{2+} can occur following even momentary bursts of activity. As such, short, rapid trains of action potentials (5 Hz, 10 s), designed to mimic the stimulus often used to initiate the afterdischarge *in situ* (Kaczmarek *et al*. 1982), were delivered to individual cultured bag cell neurons current clamped to -60 mV. Simultaneous recordings of the fluorescence signal and membrane potential revealed two phases of Ca^{2+} influx in response to this train. Initially, a distinct, fast phase of Ca^{2+} influx was observed. This was followed by a less pronounced, slower phase of Ca^{2+} elevation, which outlasted the duration of the stimulus by approximately 5 minutes (Fig. 7*A*; n=14). On occasion, a 5 Hz, 10 s train would evoke a prolonged depolarization (Hung & Magoski, 2007), that would significantly increase the time

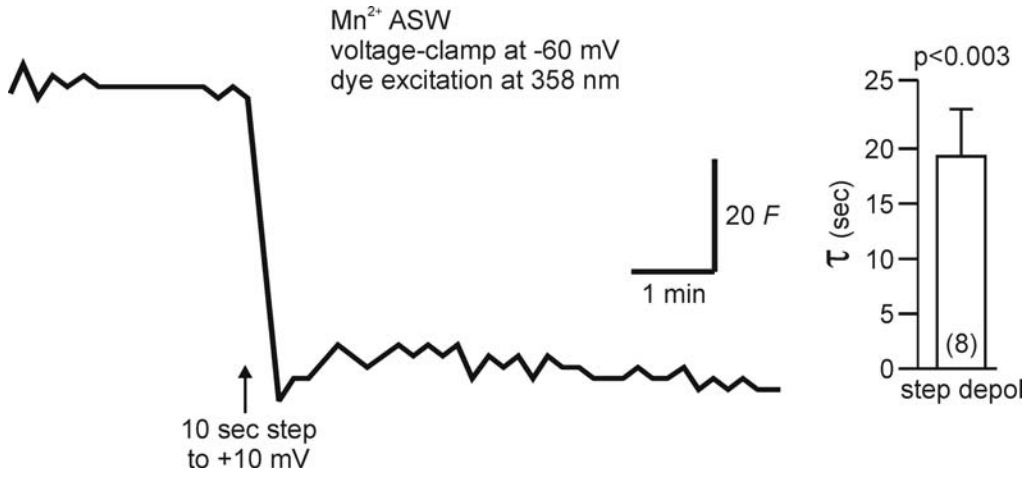

Figure 5. **Bag cell neuron Mn^{2+}-influx is detected by fura-PE3**.
Fura PE3-loaded neurons are voltage-clamped at -60 mV in ASW with Mn^{2+} substituted for Ca^{2+}. At the isosbestic point (358 nm), intensity is directly related to dye concentration, and quenching of fura by Mn^{2+}, but not Ca^{2+}, differentiates between Ca^{2+} release and Mn^{2+} influx. *Left*, Rapid quenching occurs following brief depolarization to +10 mV (at arrow) and Mn^{2+} influx through voltage-gated Ca^{2+} channels. The rate of decay is described by time constant τ. *Right*, Summary data of voltage-gated Mn^{2+} influx, which is significantly different from a mean of zero (one sample t-test).

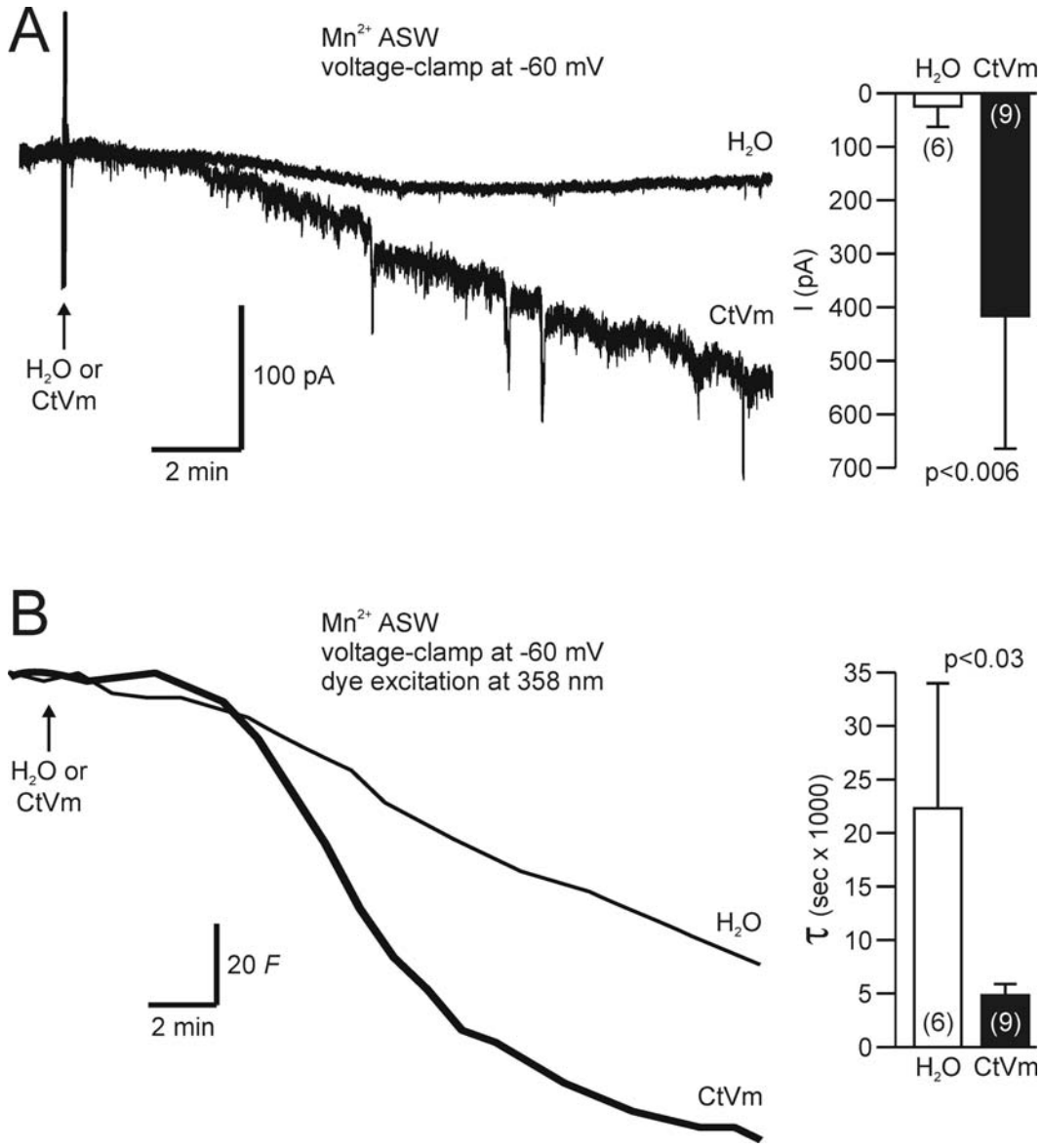

Figure 6. **Bag cell neuron Mn^{2+}-influx is enhanced by cation channel activation**. **A**, *Left,* Voltage-clamp recordings at -60 mV show that CtVm (100 μg/ml; thick line) produces prominent inward current consistent with cation channel opening, as compared to water (thin line; vehicle). *Right*, Summary data demonstrates that CtVm induces a significantly larger peak current versus control (Mann-Whitney U-test).
B, *Left*, When compared to water (thin line), CtVm (thick line) causes a more rapid decline in the fura signal. The slower decline with water is likely due to a steady leak of Mn^{2+} into the neuron. *Right*, Summary data of the time constants (τ) from single exponential fits shows that fura is quenched at a significantly greater rate following CtVm application (Mann-Whitney U-test).

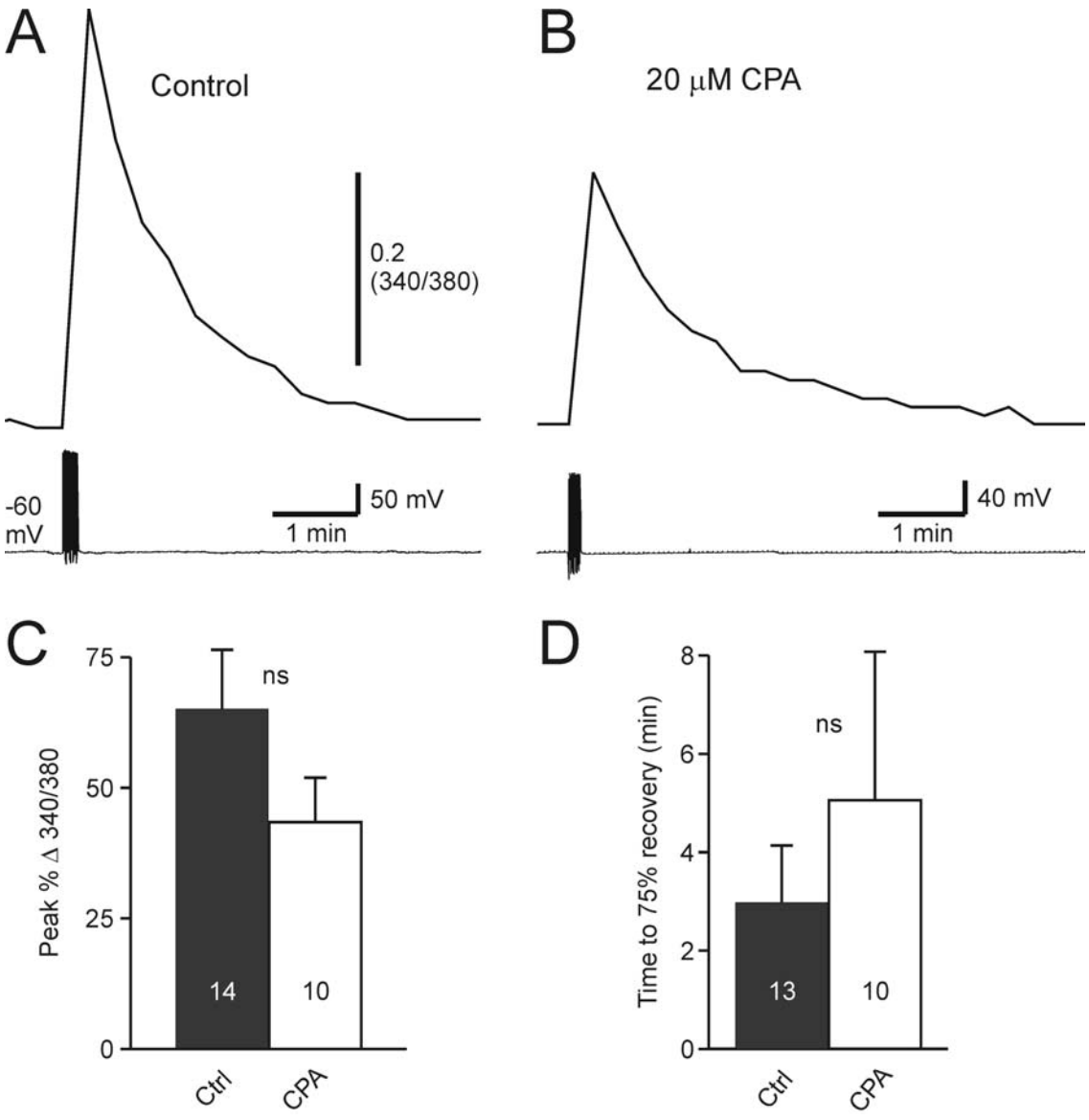

Figure 7. **Simultaneous recordings of 340/380 fura PE3 fluorescence signal and membrane potential during a 5 Hz, 10 s train of action potentials**

A, The upper panel shows Ca^{2+} influx indicated by the change in the intensity of the 340/380 ratio in nASW (11 mM Ca^{2+}). The typical recording of action potentials appears as a thick vertical bar evoked by the 5 Hz, 10 s train (lower panel).

B, The Ca^{2+} change following 25 minutes pretreatment with 20 μM CPA, an inhibitor of SERCA. Scale bar applies to both A and B.

C, The difference in peak percent change in 340/380 from control and CPA pretreated cells does not reach significance (unpaired, two-tailed t-test). For this and subsequent bar graphs, data represents the mean ± s.e.m., and the n value is indicated within the bars.

D, The time required to reach 75% recovery of baseline Ca^{2+} appears extended by CPA, however, this difference is not significant (Mann-Whitney U test).

required to return to baseline Ca^{2+} (data not shown). Care was taken to remove all neurons that displayed a prolonged depolarization from subsequent data sets.

A SERCA inhibitor, cyclopiazonic acid (CPA; 20 μM; Seidler *et al*. 1989), was added well before delivering the train to determine if the slower phase of Ca^{2+} elevation was influenced by ER Ca^{2+} uptake and/or a product of Ca^{2+} released from the ER. Following the 5 Hz, 10 s train, neurons treated with CPA appeared to have reduced peak percent change in intensity; similarly, the time to reach 75% of baseline Ca^{2+} appeared to be extended by CPA (Fig. 7*B*; n=10). However, despite these trends, neither peak percent change in intensity, nor time to reach 75 % recovery were significantly affected by CPA (Fig. 7*C* & *D*).

A potential role for mitochondria in evoking Ca^{2+} influx and/or sustaining Ca^{2+} elevation was examined by pre-treating neurons with carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 20 μM). FCCP is a protonophore that collapses the mitochondrial membrane potential, and both depletes the organelle of Ca^{2+} and prevents subsequent Ca^{2+} release from the store (Heytler & Prichard, 1962). In response to the 5 Hz, 10 s train, untreated neurons exhibited characteristic Ca^{2+} influx patterns described previously (Fig. 8*A*; n=6). As expected, pre-treatment with FCCP prior to delivering the train of action potentials resulted in robust Ca^{2+} elevation (data not shown, but see Fig. 11). In preliminary experiments, FCCP was seen to evoke a prominent depolarization coincident with the rise in Ca^{2+}. For all experiments included here, the FCCP-induced depolarization was counteracted manually by increasing, and later decreasing, delivery of hyperpolarizing current, such that the membrane potential was maintained at -60 mV throughout. Approximately 40 minutes following depletion of

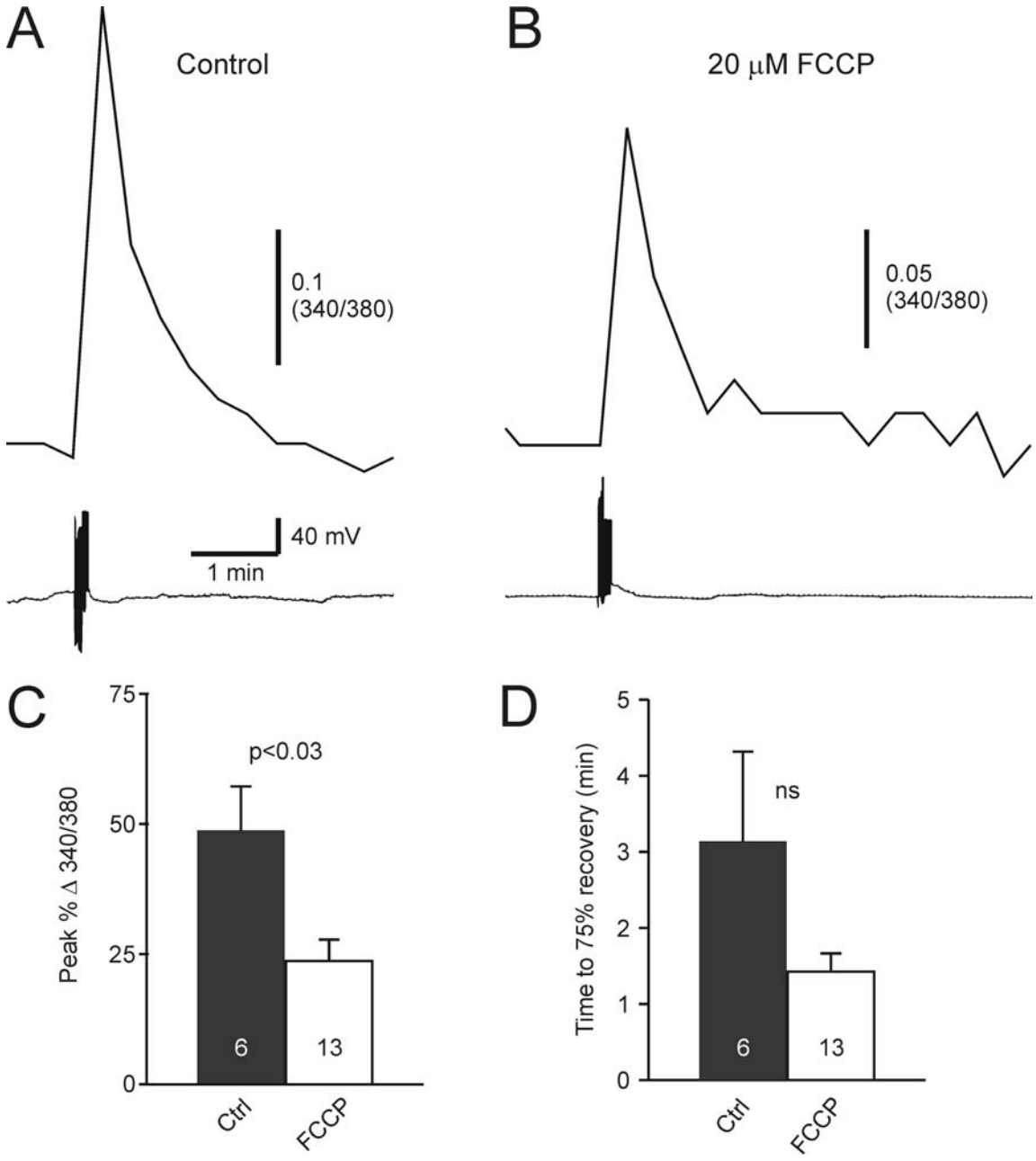

Figure 8. **FCCP lowers peak Ca^{2+} influx during a short train of action potentials**
A, Representative of the typical response in nASW to a 5 Hz, 10 s train.
B, Pretreatment with 20 μM FCCP decreases peak change in 340/380 evoked by the train. Note that the scale bars are different for the two traces.
C, Summary data for peak percent change in 340/380, indicating a significant decrease with FCCP treatment (unpaired, t-test).
D, FCCP application appears to decrease the time required to reach 75% recovery to baseline, but this difference is not significant (Mann-Whitney U test).

the mitochondrial stores with FCCP, the 5 Hz, 10 s train was delivered. Peak influx was reduced by the addition of FCCP (Fig. 8*B*; n=13), a decrease that reached the level of significance (Fig. 8*C*). Pretreatment with FCCP appeared to reduce the time to reach 75% recovery to baseline Ca^{2+}; however, this effect was not significant (Fig. 8*D*).

A prolonged train of action potentials results in Ca^{2+}-induced Ca^{2+} release

Because a relatively short stimulus failed to evoke truly prolonged changes in Ca^{2+}, a more lengthy train, one that mimicked the natural firing frequency of the fast phase of the afterdischarge, was used. Changes in intracellular Ca^{2+} which occur during the fast phase were examined with delivery of a 5 Hz, 1 min train of action potentials from -60 mV (Kaczmarek *et al*. 1982). An immediate and robust Ca^{2+} influx was observed with the delivery of the stimulus, followed by a slower and very prolonged Ca^{2+} elevation (Fig. 9*A*; n=6). This subsequent increase was suggestive of CICR, lasting several minutes.

A hallmark of CICR in most neurons is that the Ca^{2+} influx subsequently opens ryanodine receptors on the ER, thereby causing a secondary Ca^{2+} rise. A potential role for the ER store in releasing and sustaining Ca^{2+} was investigated by blocking ryanodine receptors prior to delivering the 5 Hz, 1 min train of action potentials. Pre-treatment with 100 μM ryanodine prevented further Ca^{2+} release from the ER, and the amplitude of CICR was significantly attenuated (~75% decrease) compared to control (Fig. 9*B,C*; n=5) without affecting duration.

Although caffeine, an agonist of ryanodine receptors, has been used in many other preparations (Neering & McBurney, 1984; Lipscombe *et al*. 1988; Orkand & Thomas,

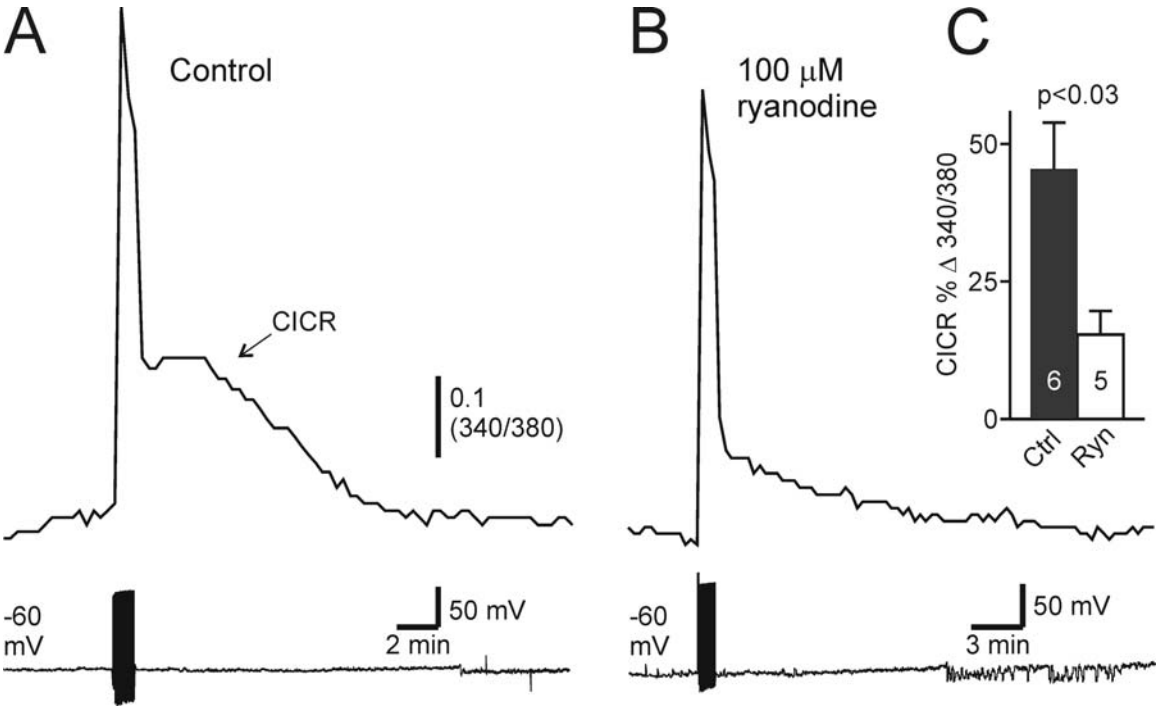

Figure 9. **Ryanodine receptors are recruited for CICR evoked by a long train of action potentials**

A, Typical response to the 5 Hz, 1 min train of action potentials, characterized by a rapid, transient Ca^{2+} elevation, followed by a slow plateau that greatly outlasts the stimulus. This second phase of Ca^{2+} elevation displays characteristics consistent with Ca^{2+}-induced Ca^{2+} release (CICR). The fluorescence ratio scale applies to panels A and B.

B, Following treatment with 100 μM ryanodine, the second phase of the Ca^{2+} elevation is markedly reduced.

C, Summary graph indicating that prior application of ryanodine significantly attenuates CICR amplitude (unpaired t test). The CICR % change 340/380 was taken as the % difference between the baseline and the most stable and largest difference during CICR.

1995; Schmigol *et al*. 1995; Usachev & Thayer, 1997; Solovyova *et al*. 2002), it was not employed in the present study. If applied at concentrations required to deplete the ER (in the mM range), caffeine strongly depolarizes bag cell neuron resting potential and inhibits voltage-dependent K^+ channels (Magoski unpublished).

CPA and FCCP deplete Ca^{2+} from distinct stores in cultured bag cell neurons

Because ryanodine receptors are typically found on the ER, this store appears to make a considerable contribution to CICR in *Aplysia* bag cell neurons. To test the involvement of this store in CICR (see next section), the SERCA blocker, CPA, was used to deplete Ca^{2+} from the ER. Initially, the ability of CPA to effectively deplete the store was tested. CPA was added to fura-loaded neurons in Ca^{2+}- free ASW. With no Ca^{2+} present in the bath, CPA addition resulted in a substantial rise in Ca^{2+} followed by a return to baseline. With subsequent addition of $CaCl_2$ to the bath, a second prominent rise in Ca^{2+} was observed (Fig. 10; n=11). This second Ca^{2+} elevation is consistent with store-operated Ca^{2+} influx, which occurs only when the ER has been depleted of Ca^{2+}. Kachoei *et al*. (2006) confirmed that this was store-operated influx and it was the result of mechanisms similar to those found in other systems (Casteels & Droogmans, 1981; Nilius, 2003; Parekh, 2003; Putney, 1986).

Given that mitochondria also sequester and release Ca^{2+} from the cytosol (Lehninger *et al*. 1967; Puskin *et al*. 1976; Akerman, 1978; Carafoli, 1979; Gunter & Pfeiffer, 1990; Colegrove *et al*., 2000a,b), I investigated the potential involvement of mitochondrial stores in CICR (see section after next). At 20 μM, FCCP effectively disables mitochondrial-storing capabilities, thus precluding CICR contribution. To determine whether the effects of CPA and FCCP depletion could be distinguished from

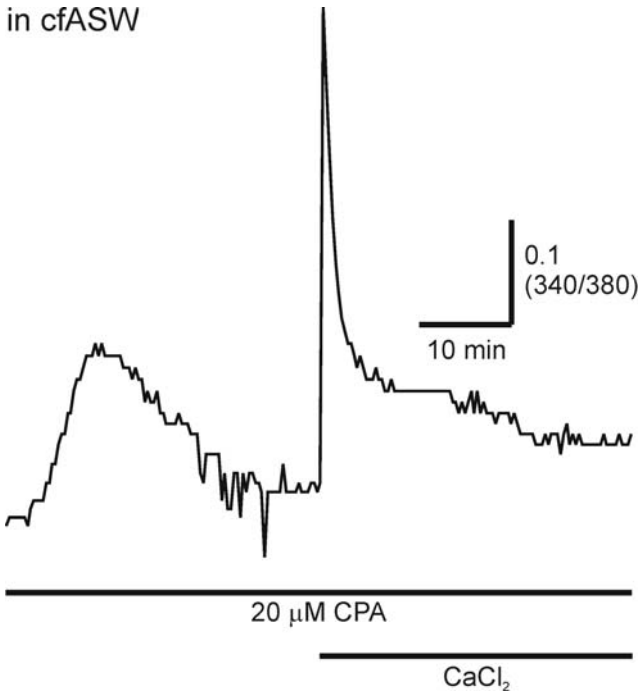

Figure 10. **CPA effectively depletes intracellular Ca^{2+} stores**
Response to Ca^{2+} addition following 20 μM CPA-induced depletion in Ca^{2+}- free ASW. In the absence of extracellular Ca^{2+}, CPA elicits an elevation in Ca^{2+}, which subsequently returns to baseline. The addition of 11 mM extracellular Ca^{2+} ($CaCl_2$) results in an additional rise in Ca^{2+}. Presumably, this second elevation is due to the prior depletion activating a store-operated pathway that allows Ca^{2+} into the neuron once it is added to the extracellular medium (see Kachoei *et al*. 2006). Representative of n=11.

one another, both agents were added in succession and the fluorescence intensity was measured. After depleting the ER stores with 20 μM CPA and allowing recovery to baseline Ca^{2+}, 20 μM FCCP was added to the bath. The addition of FCCP resulted in a second elevation in Ca^{2+} (Fig. 11; n=10). Thus signifying that the ER and the mitochondria are distinct Ca^{2+} stores.

The ER Ca^{2+} store contributes to CICR

The role of the ER in releasing and sustaining Ca^{2+} during CICR was further investigated with the addition of 20 μM CPA prior to the 5 Hz, 1 min train of action potentials. Delivery of the stimulus, in untreated neurons, evoked characteristic rapid influx and subsequent CICR (Fig. 12*A*; n=17). However, as a result of CPA-induced depletion, the amplitude of CICR was significantly attenuated by nearly half when compared to control (Fig. 12*B*,*C*; n=7). The time course of recovery to 75% of basal Ca^{2+} was not altered (data not shown).

Mitochondrial Ca^{2+} stores are essential to CICR

Although not normally considered requisite for CICR, the role of mitochondrial stores was also examined. To do so, the uncoupling agent, FCCP, was applied to the bath 20-30 minutes prior to delivering the stimulus. The depolarizing effect of FCCP was counteracted as outlined before. While delivery of a 5 Hz, 1 min train from -60 mV resulted in a robust Ca^{2+} influx and CICR in nASW (Fig. 13*A*; n=7), pre-treatment with 20 μM FCCP significantly reduced CICR amplitude (Fig. 13*B*; n=12). The time required to reach 75 % recovery to baseline Ca^{2+} was significantly shortened by FCCP (Fig. 13*D*).

Provided CPA and FCCP act on separate Ca^{2+} stores, both of which appear to be involved in CICR, the response to the long train was examined following dual depletion.

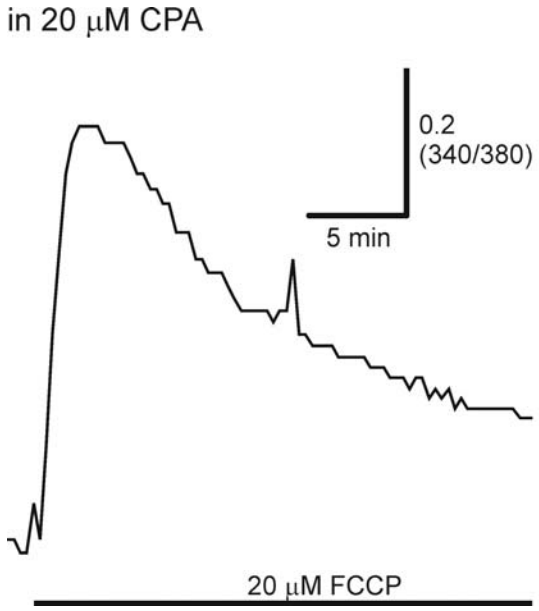

Figure 11. **FCCP depletes Ca^{2+} stores that are separate from CPA-sensitive stores** Effect of 20 μM FCCP on Ca^{2+} following depletion of the ER stores with 20 μM CPA in Ca^{2+}- free ASW. FCCP is added after the Ca^{2+} returns to baseline, following the initial rise induced by CPA (not shown). FCCP induces a second rise in Ca^{2+}, suggesting a separate FCCP-sensitive Ca^{2+} store. Representative of n=10.

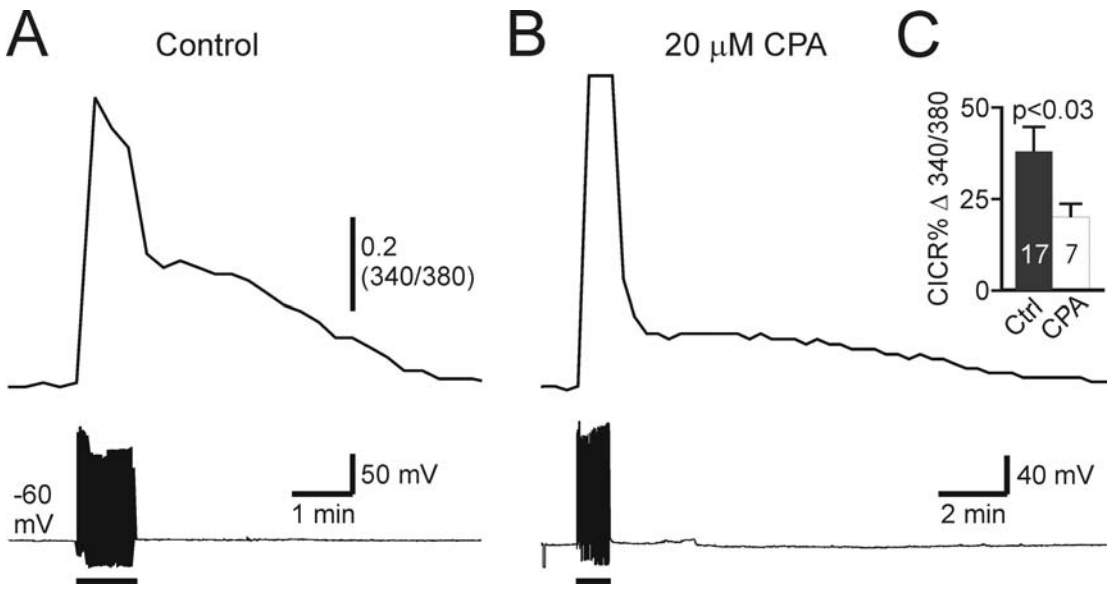

Figure 12. **Inhibition of SERCA attenuates CICR**
A, Response to the 5 Hz, 1 min train of action potentials. The scale bar for fluorescence intensity ratio applies to both A and B.
B, Prior store depletion by 20 μM CPA reduces CICR evoked by the long train.
C, Summary data showing depletion of the CPA sensitive store significantly reduces the amplitude of CICR (Mann-Whitney U test).

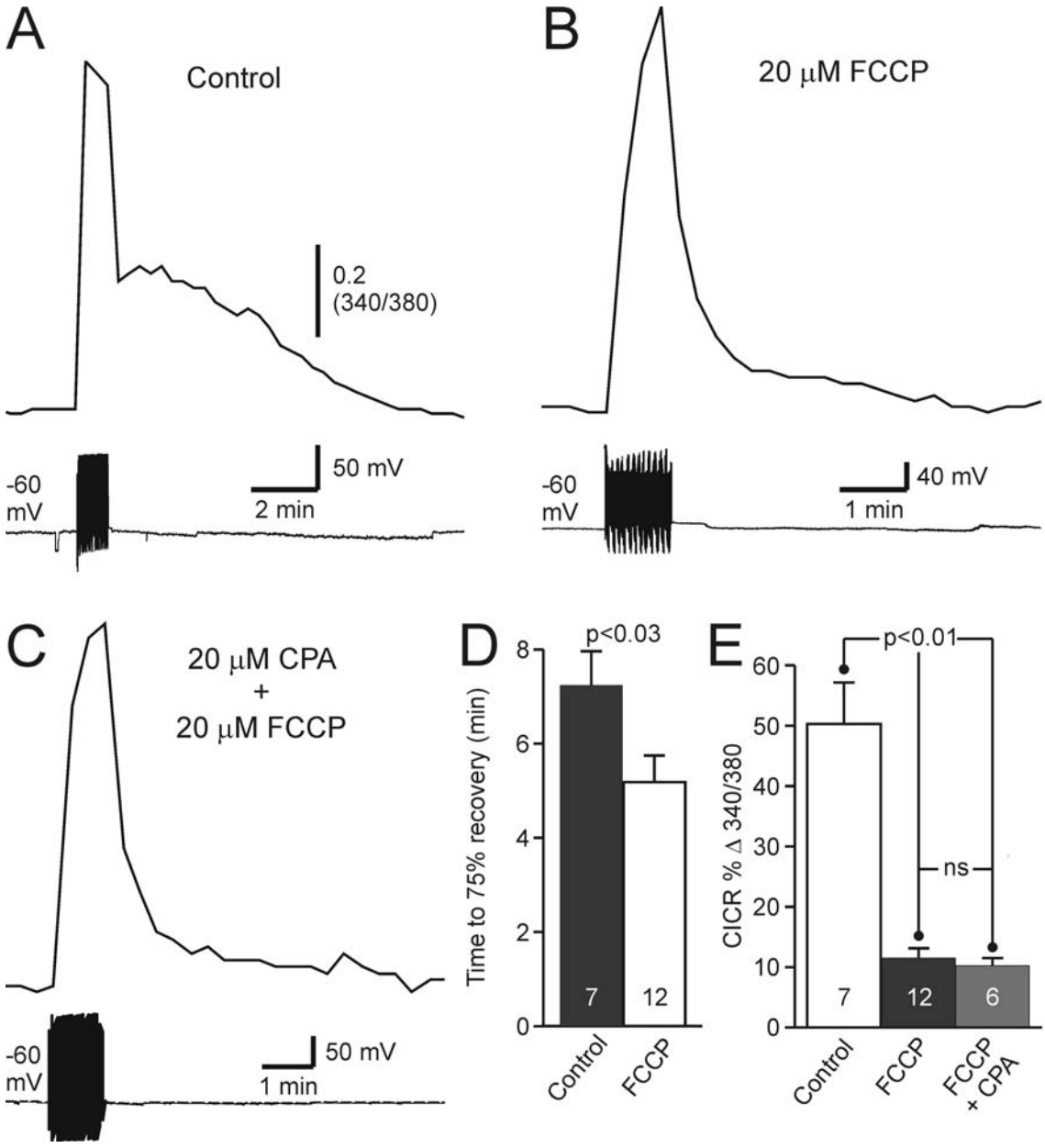

Figure 13. **Dual depletion by CPA and FCCP lacks additive inhibition of CICR**
A, CICR evoked by a 5 Hz, 1 min train. Ratio scale bar applies to panels A-C.
B, Addition of 20 μM FCCP prior to delivery of the long train clearly abates CICR.
C, Consecutive depletion by 20 μM CPA and FCCP also reduces CICR amplitude.
D, FCCP significantly reduces time to 75 % recovery (Mann-Whitney U test).
E, Summary data comparing CICR amplitude following FCCP, combined CPA and FCCP, and control. FCCP alone, and when combined with CPA, significantly reduces the amplitude of CICR. However, CICR following dual depletion does not differ from FCCP alone (Kruskal-Wallis ANOVA and Dunn's post-hoc test).

As was done before, neurons were pre-treated with 20 μM CPA to deplete the ER store. Once the fluorescence intensity returned to baseline (~20 min), FCCP was applied and the membrane potential was maintained at -60 mV. Subsequent to dual depletion, the 5 Hz, 1 min train was delivered, and the resulting CICR was significantly reduced (Fig. 13*C*; n=6). The effects of both drugs together, however, were not additive, and compared to control, the combined CPA/FCCP treatment was no more effective than FCCP alone (Fig. 13*E*; n=7; n=6; n=12).

A blocker of mitochondrial Ca^{2+} release markedly attenuated CICR

Previous studies have demonstrated that FCCP decreases ATP synthesis as a result of dissipating the mitochondrial membrane potential (Abas *et al*. 2000; Allemann & Schneider, 2000). To determine whether an associated reduction in ATP was involved in decreasing CICR amplitude, a phenomenon documented in a myocyte-derived cell line (Abas *et al*. 2000), tetraphenylphosphonium (TPP) was used as an alternative. Unlike FCCP, TPP hinders mitchondrial Ca^{2+} release without affecting ATP production (Aiuchi *et al.* 1985). TPP inhibits the Na^{+}/Ca^{2+} and H^{+}/Ca^{2+} exchange on the mitochondrial membrane, and thus blocks Ca^{2+} release (Wingrove & Gunter 1986; Karodjov *et al*. 1986). Although FCCP depletes the mitochondria of Ca^{2+}, TPP does not. Thus, testing with the latter distinguished between the influences of ATP synthesis and Ca^{2+} efflux on CICR.

In control cells, delivery of the 5 Hz, 1 min train triggered substantial Ca^{2+} influx and CICR (Fig. 14*A*; n=13). Pretreatment with 10 μM TPP had no effect on CICR (data not shown; n=5). However, 100 μM TPP significantly reduced CICR amplitude, despite having negligible effects on peak Ca^{2+} influx (Fig. 14*B*; n=5). Contrary to some

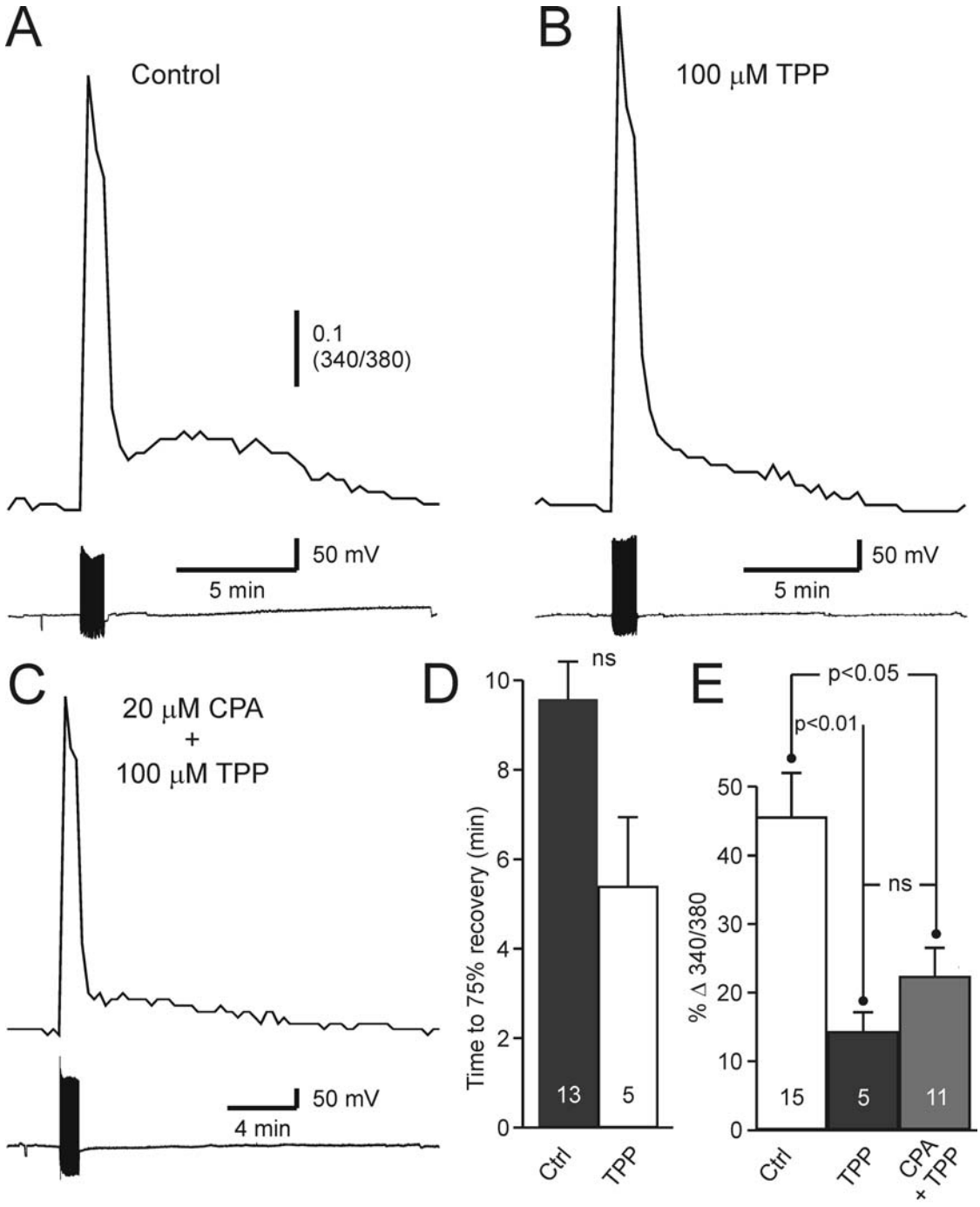

Figure 14. **TPP is as effective in reducing CICR as CPA plus TPP**
A, CICR evoked by a 5 Hz, 1 min train. Ratio scale bar applies to panels A-C.
B, Pretreatment with 100 μM TPP noticeably decreases CICR.
C, Following 20 μM CPA and TPP, CICR amplitude is also markedly reduced.
D, TPP decreases time to 75% recovery, but not significantly (Mann-Whitney U test).
E, Summary data comparing amplitude of CICR following CPA and TPP, TPP alone, and control. TPP, alone and with CPA, significantly reduces CICR, but there is no difference between TPP and TPP plus CPA (Kruskal-Wallis ANOVA and Dunn's post-hoc test).

previous reports (Aiuchi *et al*, 1985) but consistent with others (Tang & Zucker, 1997), no change in membrane potential was observed with TPP. Furthermore, TPP did not significantly change the duration of CICR (Fig 14*D*), despite a trend in the group data towards shortened recovery time.

Depletion of the ER store by 20 μM CPA, followed by 100 μM TPP treatment, also significantly attenuated CICR amplitude (Fig. 14*C*; n=11). The decrease resulting from combined CPA/TPP treatment, however, was not significantly different from TPP treatment alone (Fig 14*E*). Thus, as was the case for FCCP, elimination of the mitochondrial store effectively eliminated CICR.

Mitochondrial uniporter blocker had no effect on CICR

In addition to the exchange-mediated release of Ca^{2+}, channels on the mitochondria might also contribute to CICR. Involvement of the uniporter in sustaining CICR was investigated via treatment with ruthenium red. The uniporter is typically thought of as a pathway for Ca^{2+} to enter the mitochondria (Kirichok *et al*., 2004). It is possible that under the current conditions, Ca^{2+} may leave the mitochondria through this channel. Though some reports indicate that ruthenium red inhibits sodium-dependent calcium efflux at higher doses (Wingrove & Gunter, 1986), it is generally used to block the uniporter, because it does so with greater efficacy (Gunter and Gunter, 1994; Matlib *et al*., 1998). Without treatment, the long stimulus replicated Ca^{2+} dynamics observed under previous control conditions (Fig. 15*A*; n=9). Moreover, pretreatment with 10 μM ruthenium red had no significant effect on CICR amplitude (Fig. 15*B*, *C*; n=9) or duration (data not shown here).

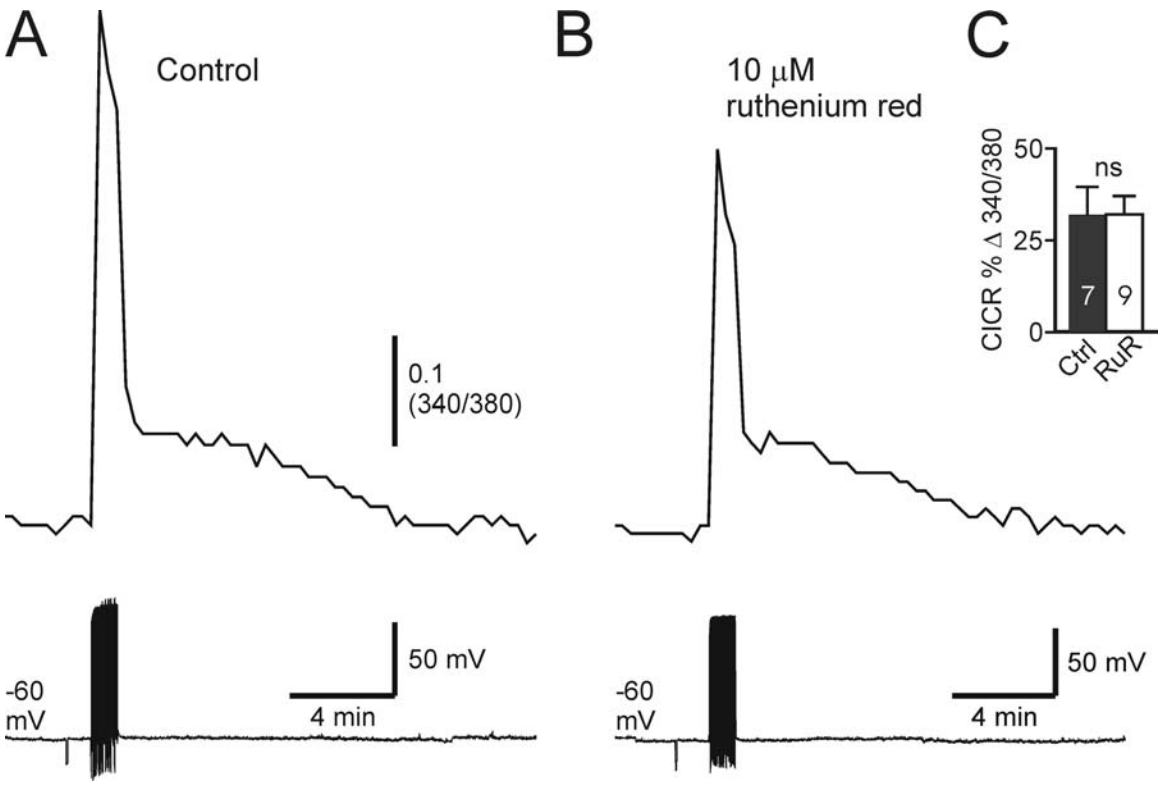

Figure 15. **CICR is not sensitive to ruthenium red**
A, As is typical in untreated bag cell neurons, a 5 Hz, 1 min train of action potentials induces CICR. The fluorescence intensity scale applies to panels A and B.
B, The response to the train following pretreatment with 10 μM ruthenium red is not markedly different from the response seen in untreated cells.
C, Summary data shows that CICR amplitude is unchanged by ruthenium red (RuR; Mann-Whitney U test).

Prior activation of PKC reduces peak Ca^{2+} influx and does not alter CICR

Approximately 5 minutes following the onset of the afterdischarge in intact bag cell neurons PKC is upregulated (Wanye *et al*., 1999). The activation of PKC in turn enhances the voltage gated Ca^{2+} current and causes an increase in action potential height (DeReimer *et al*. 1985; Strong *et al*. 1987; Conn *et al*. 1989a,b). Consequently, I tested the effects of PMA, a selective PKC activator (Castagna *et al*. 1982; Sossin & Schwartz, 1994), on CICR. Delivery of the 5 Hz, 1 min train of action potentials in untreated neurons induced robust Ca^{2+} influx and CICR (Fig. 16*A*; n=7). In neurons pre-treated with 100 nM PMA for 25 minutes prior to the stimulus, peak Ca^{2+} influx during the train was modestly reduced by ~25 %, while CICR amplitude was unchanged (Fig. 16*B-D*; n=10). The lowering of action potential-induced Ca^{2+} influx by prior activation of PKC may be due to enhanced Ca^{2+}-induced inactivation of the Ca^{2+} current.

Changes to Ca^{2+} during a simulated afterdischarge

In examining the responses to the 5 Hz, 1 min train, the Ca^{2+} dynamics associated with the fast phase of the afterdischarge were studied in isolation. Physiologically, however, the fast phase is followed by a prolonged period of slow firing (1 Hz, 30 min). Thus, the afterdischarge firing pattern was simulated by delivering a compound train consisting of a 5 Hz, 1 min train, immediately followed by a 1 Hz, 30 min train (Fig 17*C*). Delivery of the simulated afterdischarge produced robust Ca^{2+} influx as before. Apparent CICR was sustained for the duration of the stimulus and gradually recovered to baseline Ca^{2+} following termination of the train (Fig. 17*A*; n=10). Pre-treatment with TPP did not reduce the amplitude of CICR (Fig 17B; n=9). This lack of an effect was

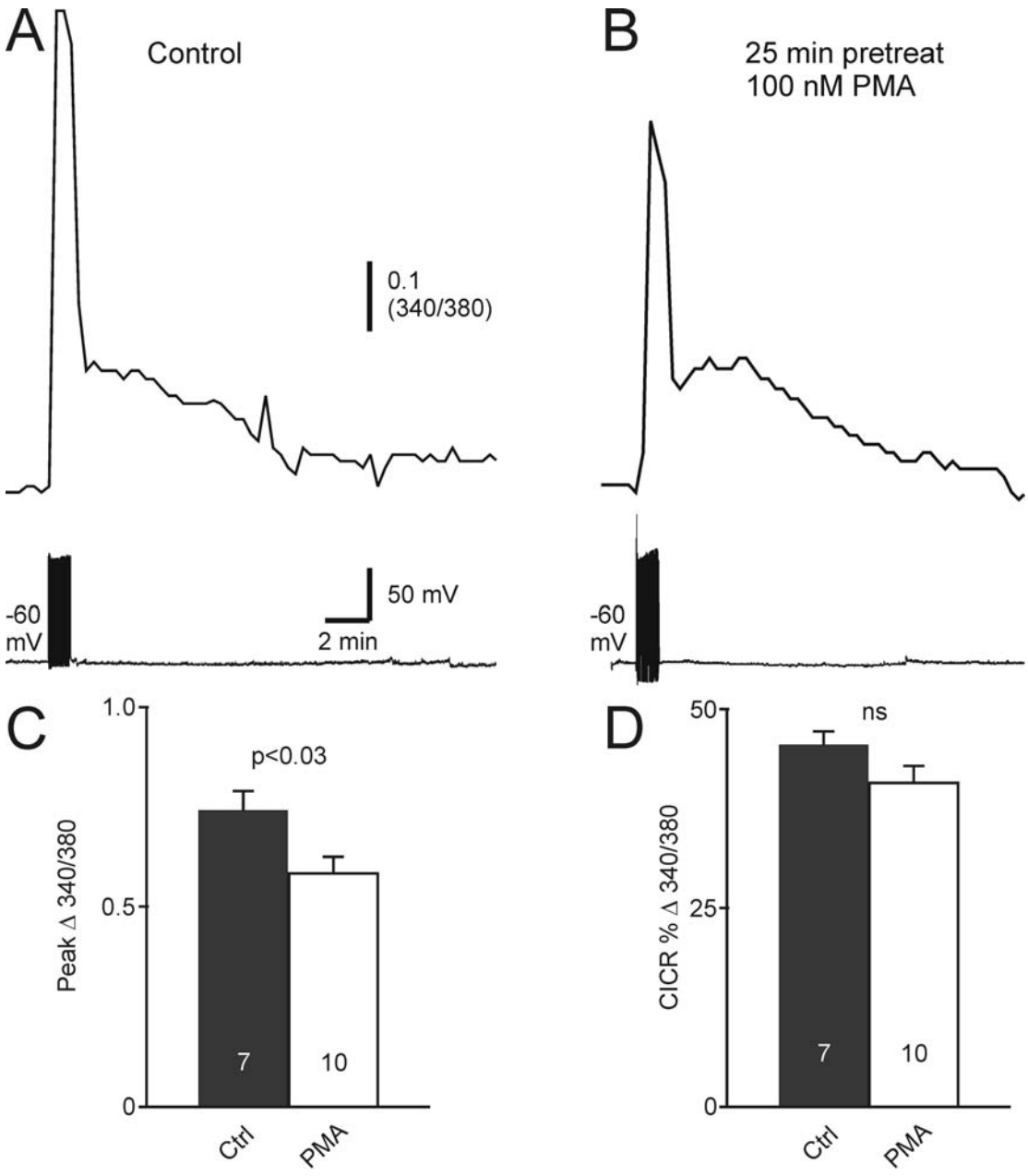

Figure 16. **Peak Ca^{2+} influx is reduced, but CICR amplitude is unaffected by prior PKC activation**

A, Response evoked by a 5 Hz, 1 min train of action potentials. The scale bar for fluorescence intensity applies to both panels A and B.

B, A 25 minute pretreatment with 100 nM PMA does not alter CICR amplitude. However, PKC activation does decrease the amplitude of initial Ca^{2+} influx.

C, Peak Ca^{2+} influx is significantly reduced by PMA (Mann-Whitney *U* test).

D, Summary data shows that CICR amplitude is not altered by PMA (Mann-Whitney U test).

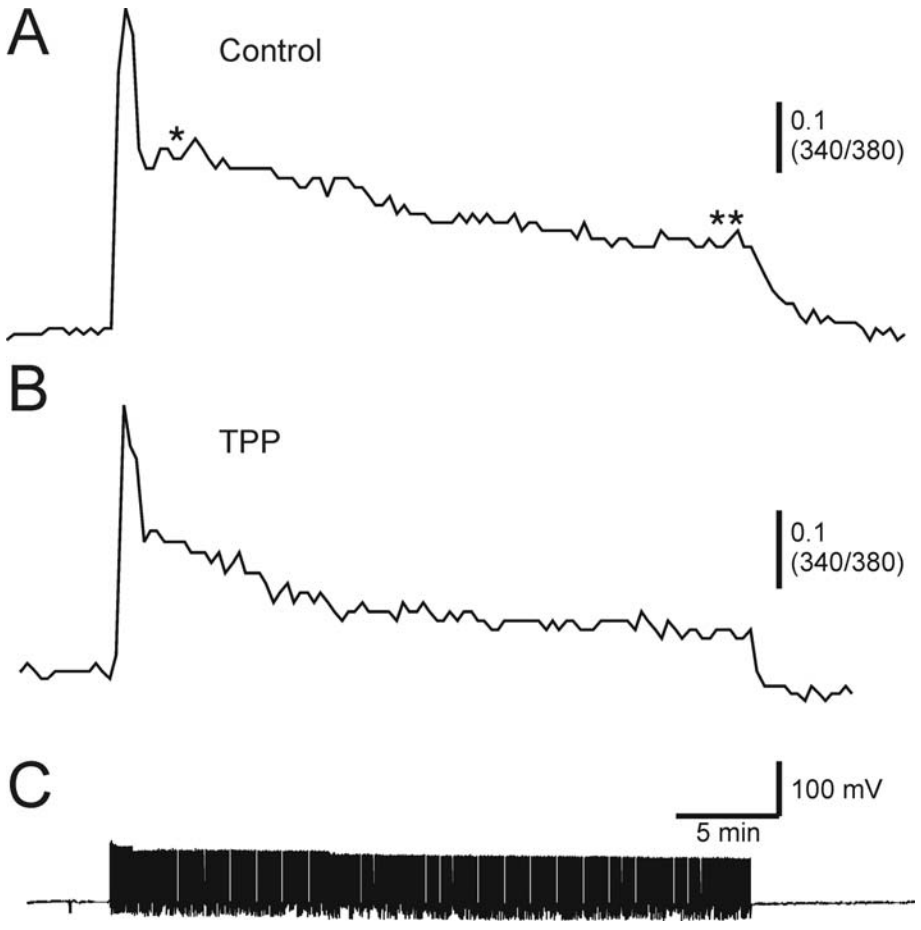

Figure 17. **TPP does not reduce Ca^{2+} influx triggered by a simulated afterdischarge** **A,** Representative of Ca^{2+} measured in response to a compound train of action potentials simulating the afterdischarge (5 Hz, 1 min continuous with 1 Hz, 30 min). Data points from two periods during the 1 Hz, 30 min phase of the train, are used to determine the Ca^{2+} amplitude during the simulated AD. These phases are marked early (*) and late (**), which correspond with an initial stage and the conclusion of the 1 Hz, 30 min train. **B**, The Ca^{2+} pattern observed with 100 μM TPP treatment is similar to that of control. **C,** A typical current clamp trace taken from control conditions. The scale bar for time applies to all panels.

observed at both the peak of the Ca^{2+} elevation during the 30 min, 1 Hz phase of the train, and at the end of the compound train (Fig. 17*A*,*B*). In the prior experiment involving PKC activation, PMA was added well before the train was delivered. However, as mentioned, in the intact cluster PKC activity is not initiated until 5 minutes subsequent to the onset of the afterdischarge (Wayne *et al*. 1999). Thus, in an effort to perpetuate afterdischarge-like kinetics for PKC activation, PMA was applied just 10 min prior to the compound train (Fig. 18). Conn *et al*. (1989b) showed that bath applied PMA increases Ca^{2+} current and action potential height within 10 minutes. Treatment with 100 nM PMA 10 minutes prior to the simulated afterdischarge train enhanced action potential height 5 minutes into the 30 min, 1 Hz phase of the train. As action potentials increased in height so too did Ca^{2+} influx (n=7).

The summary data (Fig. 19*A*,*B*) show that 100 μM TPP did not significantly alter the Ca^{2+} change at the early or late portion of the slow phase train. On the other hand, PMA significantly enhanced the Ca^{2+} elevation, both at the early and late stage of the 30 min 1 Hz train of action potentials. Unlike TPP, 100 nM PMA significantly prolonged the recovery to baseline duration (Fig. 19*C*).

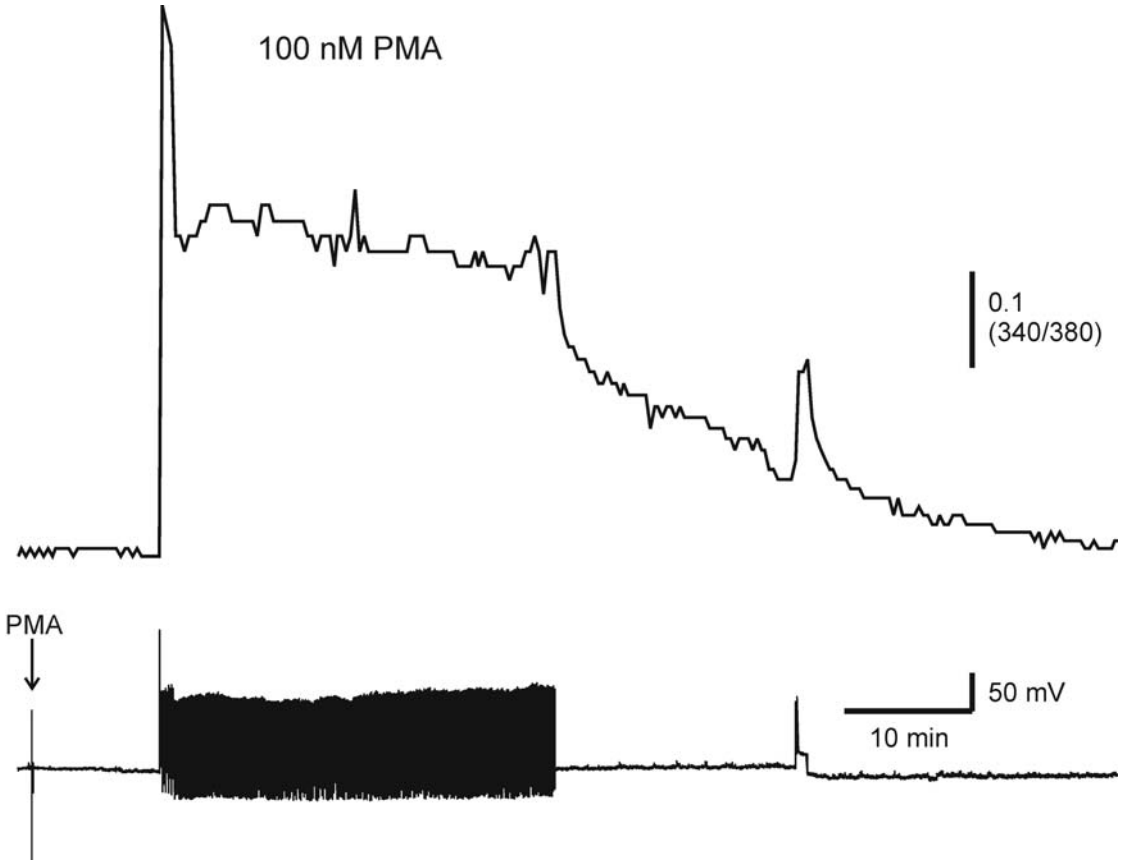

Figure 18. **Ca^{2+} is enhanced by a simulated afterdischarge delivered shortly after PKC activation**

Effect of 100 nM PMA addition (at arrow), 10 min prior to the delivery of the compound train. PMA results in a greater Ca^{2+} influx, increased Ca^{2+} during the slow phase, and a much longer time to recovery after the train. A short spontaneous burst of action potentials occurs ~15 min post train, which alters fluorescence initially but has no apparent effect on time to recovery. This effect is sometimes seen with prolonged exposure to PMA (Tam & Magoski unpublished).

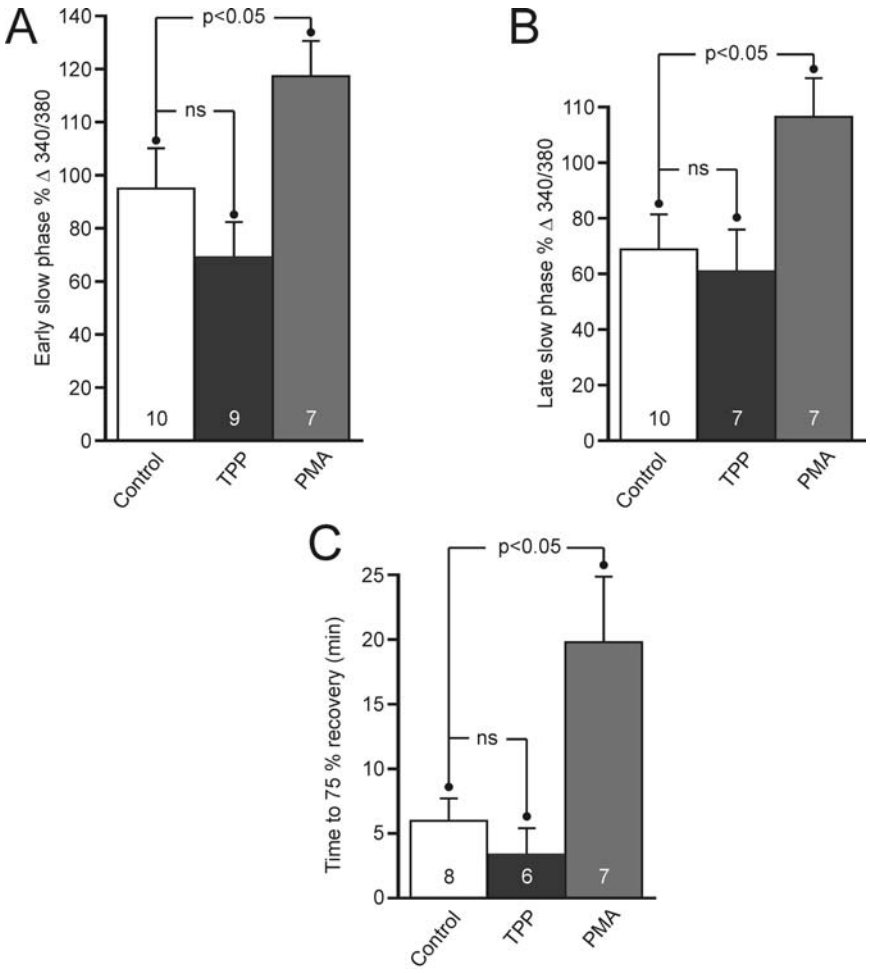

Figure 19. **Enhanced Ca^{2+} following PKC activation**
A, Summary data comparing the percent change in Ca^{2+} amplitude taken from the early plateau stage. During this period, TPP treatment does not affect the magnitude of the Ca^{2+} plateau, while PMA results in a significant increase in Ca^{2+} (Standard ANOVA and Dunnett's multiple comparison test).
B, The percent change in Ca^{2+} amplitude taken from the late stage, where again, TPP treatment does not alter the response significantly. PMA, on the other hand, causes a significant increase in Ca^{2+} (Standard ANOVA and Dunnett's multiple comparison test).
C, Summary data for the time to reach 75 % recovery to baseline. Though often more abrupt, the recovery following TPP is not different from control. Conversely, the addition of PMA significantly lengthens the time to reach 75 % recovery (Standard ANOVA and Dunnett's multiple comparison test). In two controls, before 75% recovery was reached, there was a computer error is saving the data or the impalement was lost, respectively. Thus, their n-values decrease from 10 in panel B to 8 in panel C. In three of the initial TPP treated neurons, one developed pathologically large Ca^{2+} oscillations half way through the slow phase, another lost the impalement shortly before the end of the train, and a third developed an additional rise in Ca^{2+} after the train but in the absence of any further spiking. Hence, the TTP n-values decrease from 9 in panel A to 7 in panel B and 6 in panel C.

Chapter 4: Discussion

Following a brief synaptic stimulus, the bag cell neurons of *Aplysia* undergo a characteristic sequence of action potentials, known as the afterdischarge. Delivery of a 4-6 Hz, 10 s train is typically used to evoke the afterdischarge in intact clusters (Fisher *et al*. 1994; Kaczmarek *et al*. 1982; Magoski & Kaczmarek, 2005; Zhang *et al*. 2002). This pattern consists of two phases of activity: a short period of high frequency firing (~ 5 Hz, 1 min), and a prolonged period of low frequency firing (~ 1 Hz, 30 min; Kaczmarek *et al*. 1982). Initiation of the afterdischarge results in a rapid increase in intracellular Ca^{2+} (Fisher *et al.* 1994), leading to the release of ELH, which initiates reproductive behaviours completed by egg deposition (Stuart *et al.* 1980; Loechner *et al*. 1990).

In the presence of Ca^{2+}, ELH release can be elicited by high K^{+}-induced depolarization (Arch, 1972a,b). Yet, secretion can occur during changes in intracellular Ca^{2+} that outlast action potential firing (Michel & Wayne, 2002). As in other biological systems, Ca^{2+} influx in bag cell neurons occurs largely through voltage-gated Ca^{2+} channels (see below). However, Ca^{2+} levels are further regulated by intracellular Ca^{2+} stores, as demonstrated by Fisher *et al*. (1994), who showed that intracellular Ca^{2+} increased during action potential firing in the presence of Ba^{2+}, suggesting Ca^{2+} was released from stores by Ba^{2+} influx.

Ca^{2+} channels and Ca^{2+} influx in bag cell neurons

The primary route of Ca^{2+} influx, through voltage-gated Ca^{2+} channels, is a function of the magnitude and duration of the depolarizing stimulus, which under physiological conditions is the action potential (Shmigol *et al*. 1995; Park & Dunlap, 1998; Klein & Kandel, 1978; Eliot *et al.* 1993; Augustine *et al.* 2003; Hung & Magoski,

2007). Action potentials in bag cell neurons undergo frequency-dependent broadening and thus, allow progressively more Ca^{2+} to enter as a burst continues (Dudek & Kossatz, 1982; Fink *et al*. 1988; Fisher *et al*. 1994; Quattrocki *et al.* 1994). Just as Ca^{2+} entry is highly regulated, the removal of Ca^{2+} is also tightly controlled (Clapham, 1995; Kim *et al*. 2005; Meldolesi, 2001; Verkhratsky, 2005). In bag cell neurons, concomitant with a short train (5 Hz, 10 s), intracellular Ca^{2+} increases appreciably and is presumably removed by plasma membrane pumps and exchangers (Knox *et al*. 1996).

Organelles, such as the ER, may aid in the removal of intracellular Ca^{2+}. Yet, in the present study, it appears that the ER does not rapidly take up Ca^{2+} during brief, modest increases, given that there was no significant effect of the SERCA blocker, CPA, on the amplitude of intracellular Ca^{2+} during the short train. Furthermore, CPA did not significantly prolong the time to reach 75 % recovery to baseline Ca^{2+}, unlike that observed in leech neurons (Beck *et al*. 2001). It is possible that the bag cell neuron ER takes up Ca^{2+} only when intracellular levels reach some threshold. As Ca^{2+} is only modestly increased during a short stimulus, SERCA is not recruited. That said, the prolonged time to recovery with CPA in some neurons, which accounts for the large standard error in that data set, may suggest that some SERCA are activated if Ca^{2+} levels reach threshold in their immediate vicinity.

Surprisingly, the addition of FCCP decreased intracellular Ca^{2+} during the short train, rather than increasing levels following the collapse of V_{mit} and disablement of Ca^{2+} uptake by the mitochondria. Perhaps the Ca^{2+} released from mitochondria after FCCP treatment initiated a process whereby Ca^{2+} channels were altered or Ca^{2+} removal systems augmented. Alternatively, functioning mitochondria may be important for

shaping the initial activation of the Ca^{2+} current, as is the case for store-operated Ca^{2+} channels (Gilabert & Parekh, 2000).

At the onset of the afterdischarge, Ca^{2+} also enters through other channel types, including the non-selective cation channels that provide the necessary depolarizing drive (Wilson & Kaczmarek, 1993; Wilson *et al*. 1996; Hung & Magoski, 2007). Results from the Mn-quench experiments suggest that one of these cation channels (triggered by CtVm) is also permeable to Ca^{2+}. Furthermore, single-channel work on said conductance and its permeability support this conclusion (Magoski & Geiger, 2006). This receptor-operated route of Ca^{2+} influx, which likely occurs through second messenger activation of the channel (Wilson & Kaczmarek, 1993; Magoski *et al*. 2002), seems to provide both positive and negative feedback for the afterdischarge; upon entry, Ca^{2+} appears to activate this channel (Lupinsky & Magoski, 2006) and initiates processes crucial to the refractory period (Magoski *et al*. 2000).

Bag cell neuron CICR

The plateau of Ca^{2+} following a prolonged burst of action potentials in bag cell neurons is consistent with CICR observed in other preparations. This is to say, it is sensitive to ryanodine, which acts on receptors predominantly in the ER membrane (Berridge, 1998; Meldolesi, 2001; Bardo *et al*. 2006). Ryanodine is unusual in that is has dual effects. At low concentrations (usually 10 nM), it is an agonist, but at concentrations greater than 10 μM, it is an antagonist (Meissner, 1986). At 100 μM or above, ryanodine profoundly blocks CICR in leech Retzius neurons (Trueta *et al.* 2004), salamander photoreceptors (Suryanarayanan & Slaughter, 2006), and rat DRG neurons (Shmigol *et al*. 1995). CICR is also suppressed by ryanodine at 50 μM in frog

sympathetic neurons (Hua *et al*. 1993) and 20 μM in *Hermissenda* photoreceptors (Kawai *et al.* 2004). Work done on rat DRG neurons showed that following voltage-gated Ca^{2+} influx, Ca^{2+} within the ER (monitored by mag fura-2) dropped while cytosolic Ca^{2+} rose, a response that was sensitive to ryanodine (Solovyova *et al*. 2002). The effect of 100 μM ryanodine in the present study is in agreement with RyRs having a role in generating CICR in bag cell neurons.

Intracellular Ca^{2+} stores within bag cell neurons are also sensitive to the ryanodine receptor agonist, chloroethylphenol (Zorzato *et al*. 1993). CPA-sensitive Ca^{2+} stores and chloroethylphenol sensitive stores overlap almost completely (Kachoei *et al*. 2006). This suggests that CPA should deplete stores that release Ca^{2+} through ryanodine receptors subsequent to Ca^{2+} influx. Since depletion of CPA-sensitive stores and subsequent reduction in CICR is much like the effect of ryanodine on CICR, it upholds this conjecture. The CPA result is further indicative of a RyR-ER pathway, and is similar to that observed in other neurons, including those from *Helix* (Orkand & Thomas, 1995), rat DRG (Usachev & Thayer, 1997), rat hippocampus (Emptage *et al*. 1999), and rat thalamus (Richter *et al*. 2005).

A short train of action potentials (5 Hz, 10 s) failed to initiate CICR in bag cell neurons. CICR was initiated only in response to a sustained period of firing (5 Hz, 1 min). Schmigol *et al*. (1995) reported similar results in rat DRG neurons, where CICR could not be elicited by single action potentials or short bursts. Thus, CICR in bag cell neurons appears to be a cross between an all-or-none (Usachev & Thayer, 1997) and a burst-dependent response (Smith *et al*. 1983; Neering & McBurney, 1984; Richter *et al*. 2005). This is unlike certain reports, where short bursts or single action potentials were

enough to initiate CICR (Gorman & Thomas, 1980; Hua *et al*. 1993; Orkand & Thomas 1995; Solovyova *et al*. 2002). The response displayed by the bag cell neurons seems entirely appropriate, as secretion of ELH and other peptides should be initiated precisely when bag cell neurons are sufficiently stimulated. Only when the afterdischarge is initiated and underway, following the fast phase, should CICR become engaged. Initiation of CICR with just a brief synaptic input, without the guarantee of triggering and sustaining the afterdischarge, could lead to unregulated or erroneous Ca^{2+} release with inappropriate effects.

Dependence of bag cell neuron CICR on mitochondrial Ca^{2+}

Depletion of the mitochondrial store with FCCP, or preclusion of mitochondrial Ca^{2+} release with TPP, nearly eliminates CICR in bag cell neurons. Yet, simultaneous ER depletion by CPA does not reduce CICR further. Thus, although supported by Ca^{2+} from the ER, CICR appears to be dependent on mitochondrial Ca^{2+} handling. I propose that these features render bag cell neuron CICR unique.

FCCP depletes mitochondria by collapsing the mitochondrial membrane potential, preventing further Ca^{2+} release (Heytler & Prichard, 1962). Previous studies with JC-1, a dye used to measure V_{mit}, revealed that FCCP depolarizes V_{mit} in HeLa cells (Collins *et al*. 2000). Heytler and Prichard (1962) initially reported that at 100 nM, FCCP lowered oxidative phosphorylation to levels approaching zero in beef heart mitochondria. Thus, the precise effect of FCCP on CICR can be uncertain, as FCCP will also reduce ATP production.

The use of FCCP also raises the question of whether Ca^{2+} release from the ER is dependent on ATP and/or mitochondrial Ca^{2+}. TPP was thus employed to distinguish

between these two possibilities, as it blocks Ca^{2+} release from mitochondria (Wingrove & Gunter, 1986) with little effect on ATP production (Aiuchi *et al*. 1985). Although TPP blocks Na^{+}-dependent Ca^{2+} release with an IC_{50} of 10 nM (Karadjov *et al*. 1986), 10 μM is required for 100 % and 50 % block of Na^{+}-dependent and independent release, respectively (Wingrove & Gunter, 1986). As such, I used TPP at 100 μM to prevent CICR in bag cell neurons. The FCCP and TPP results, which are essentially identical, suggest that mitochondrial Ca^{2+} release is required for bag cell neuron CICR.

There is evidence from other preparations that the mitochondria can influence CICR. For example, when Werth and Thayer (1994) uncoupled mitochondria and prevented electron transport, it blocked a depolarization-induced Ca^{2+} release very similar to bag cell neuron CICR. They suggest that mitochondria take up Ca^{2+} as it enters during the depolarization and then release it during a plateau phase. Similarly, Friel and Tsien (1994) showed in frog sympathetic neurons that high K^{+} caused Ca^{2+} influx, presumed mitochondrial filling, and subsequent mitochondrial release of Ca^{2+} in a pseudo CICR-like fashion. This sustained Ca^{2+} release was prevented by FCCP, modestly antagonized by caffeine, but not altered by ryanodine. Friel and Tsien (1992) also observed true CICR in frog sympathetic neurons, one that was blocked by depletion with caffeine, yet only weakly sensitive to ryanodine. They concluded that a large Ca^{2+} influx engaged mitochondria, while a modest Ca^{2+} influx engaged ER, with no interaction existing between the two.

However, the activation of CICR in bag cell neurons hinges on the mitochondrial store, and thus, as proposed by Jackson and Thayer (2006), raises the possibility of an interaction between mitochondrial and ER stores. In rat DRG neurons, caffeine

application induced rhythmic Ca^{2+} release through RyRs that was prevented, in a fractional manner, by FCCP and a mitochondrial Ca^{2+} release blocker. This suggested that the RyR, and ultimately CICR, is influenced by both Ca^{2+} release from mitochondria and ATP levels. But is this the case for CICR in bag cell neurons?

There are two plausible explainations for the apparent dependence of bag cell neuron CICR on mitochondria. Firstly, Ca^{2+} influx during the stimulus may load the mitochondria with Ca^{2+}, which would be subsequently released through one or both exchanger pathways. This Ca^{2+} would then activate ryanodine receptors, which in turn cause Ca^{2+} release from the ER (CPA and ryanodine sensitive). Depletion of the ER does not completely eliminate CICR, since mitochondrial release contributes to the plateau and activates RyRs. Thus, depletion (with FCCP) or elimination (with TPP) of the mitochondria impedes both mechanisms. Secondly, Ca^{2+} influx during the 5 Hz, 1 min train could simply activate, through a currently unknown mechanism, the mitochondrial exchangers, which would release Ca^{2+} and initiate CICR as described.

Although the depletion of mitochondria by FCCP might have been accompanied by a change in ATP levels, which could in turn affect RyRs, results from TPP application do not support this suggestion. Instead, the TPP effect further supports the initial postulate. Thus, although similar to that in rats and frogs, CICR in bag cell neurons is novel in that it appears to have absolute dependence on mitochondria. Seemingly for Ca^{2+} buffering and release, the mitochondria, with a subsequent contribution from the ER, initiate and support CICR without ATP-RyR interactions. This represents a departure from the classic model of CICR (compare Fig. 1 with Fig. 20).

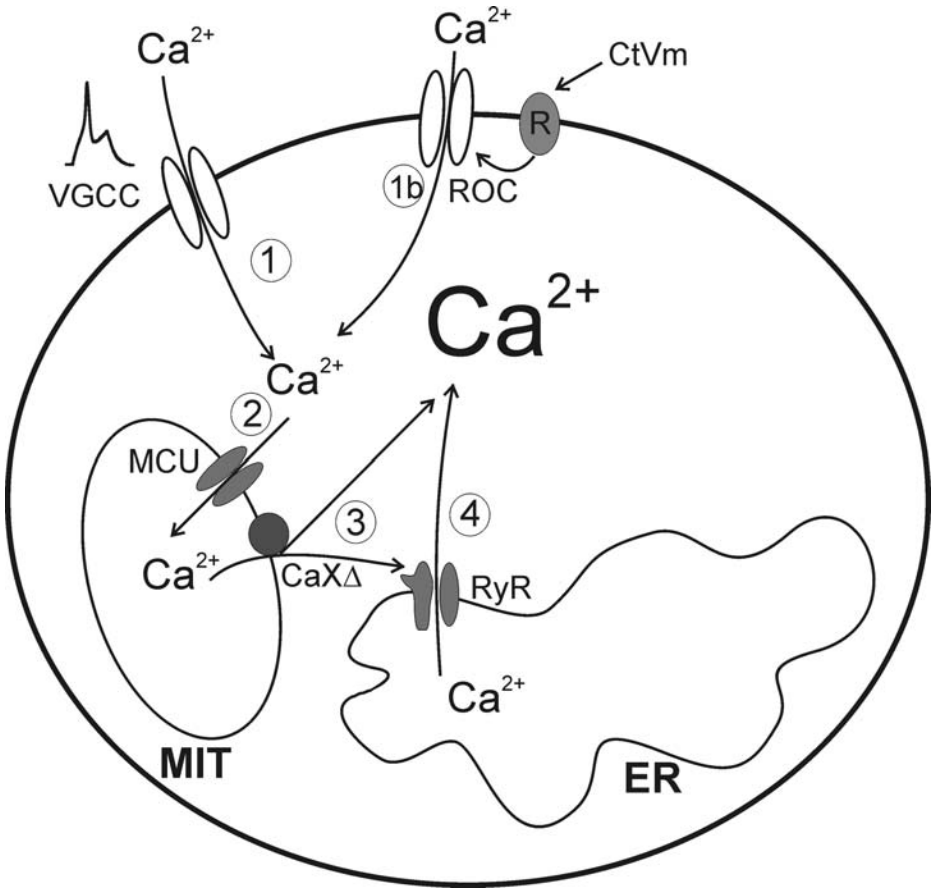

Figure 20. **Bag cell neuron CICR**
An updated schematic for the regulation of intracellular Ca^{2+} and initiation of CICR-based on the bag cell neurons. Ca^{2+} influx occurs via voltage-gated Ca^{2+} channels (VGCC; step 1) and upon entry, Ca^{2+} is pumped into the mitochondria by the mitochondrial uniporter (MCU; step 2). Ca^{2+} is released from the mitochondria though cation exchangers on the mitochondrial membrane (step 3). Subsequently, the Ca^{2+} released may stimulate RyRs on the ER membrane, producing CICR (step 4). In addition, Ca^{2+} release from mitochondria directly contribute to CICR. Ca^{2+} influx also occurs via receptor-operated activation of a non-selective cation channel following CtVm application (ROC; step 1b).

Interactions between mitochondria and other Ca^{2+} channels on the ER have already been recognized. For example, in HeLa cells, FCCP prevents IP_3-dependent liberation of Ca^{2+} from the ER in response to metabotropic agonists, despite not changing the response to the ER depleter, thapsigargin (Collins *et al*. 2000). These authors also demonstrated, using mitochondrial-targeted aequorin, that metabotropic agonists increased mitochondrial Ca^{2+} after increasing cytosolic Ca^{2+} via IP_3. Furthermore, histamine-induced Ca^{2+} oscillations in HeLa cells are due to IP_3-mediated Ca^{2+} release from the ER, with that Ca^{2+} being subsequently taken up by the mitochondria and re-released to re-activate IP_3Rs (Ishii *et al*. 2006).

CICR during afterdischarge-like spiking

When bag cell neurons are stimulated from rest by either the stimulus inducing the afterdischarge (5 Hz, 10 s train) or the simulated firing frequency of the fast phase (5 Hz, 1 min train), Ca^{2+} influx occurs through the basal Ca^{2+} channel (12 pS; DeRiemer *et al*. 1985). Within the first few minutes of the slow phase, however, PKC is activated (Wayne *et al*. 1999) and consequently a PKC sensitive channel (24 pS) is inserted into the plasma membrane (DeRiemer *et al*. 1985; Conn *et al*. 1987; Strong *et al*. 1987). This results in a progressive increase in action potential height (Kaczmarek *et al*. 1982, Conn *et al*. 1987, Strong *et al*. 1987).

Initially, attempts at enhancing Ca^{2+} influx during the fast phase-like stimulation of 5 Hz, 1 min with PMA pre-treatment were unsuccessful. Instead, PMA produced a smaller Ca^{2+} elevation. Likely, intracellular Ca^{2+} was lowered because Ca^{2+}-dependent inactivation of the current (Tillotson, 1979) was increased due to a combination of rapid stimulation and the PKC-mediated enhancement of the current itself. This outcome fails

to replicate natural phenomena, as these conditions are unrealistic; PKC activity is not enhanced during the fast phase of the afterdischarge, but instead during the slow phase (Wayne *et al*. 1999). Thus, in later experiments, PMA application was timed appropriately, at 10 minutes prior to the simulated afterdischarge, such that PKC activity would be elevated several minutes into the slow phase. PKC activation resulted in a prominent increase in the overall amount of Ca^{2+} in the cell during the slow phase. Moreover, post-train recovery time was lengthened by PMA, suggesting that CICR may have occurred subsequent to the end of the afterdischarge. While these experiments were ongoing, parallel work in the lab showed that PMA readily increased Ca^{2+} current, as assayed under voltage-clamp with ionic isolation (Tam & Magoski unpublished), in a manner consistent with DeRiemer *et al*. (1985).

Modulation of bag cell neuron CICR was investigated further with the delivery of the simulated afterdischarge under control conditions versus pretreatment with TPP. While the prolonged Ca^{2+} elevation seen during the compound train was clearly reduced in some cases, overall it was unaffected by TPP. Unexpectedly, post train recovery time was unchanged by TPP pretreatment. It is conceivable that mitochondrial-mediated CICR is not active during the slow phase, or it is undetected due to the overwhelming Ca^{2+} influx that occurs during 1 Hz firing. I could not test the effect of TPP and PMA together because the drugs caused the action potentials to become extraordinarily broad.

Role for CICR in bag cell neurons

The fact that bag cell neurons undergo voltage-gated Ca^{2+} influx, provokes questioning the purpose of CICR. Possibly, CICR is needed to amplify or intensify ELH secretion, as similar phenomena have been observed previously in both vertebrates and

invertebrates. For example, CICR augments the sustained release of acetylcholine in *Aplysia* buccal neurons (Mothet *et al*. 1998, Chameau *et al*. 2001), glutamate at salamander photoreceptor synapses (Suryanarayanan & Slaughter, 2006), and secretion of insulin from a beta cell line (Kang & Holz, 2003). In addition, blocking CICR in leech Retzius neurons reduces a portion of serotonin secretion (Trueta *et al*. 2004).

Bag cell neuron CICR may also be necessary for the activation of membrane conductances contributing to the depolarizing drive for the afterdischarge. This would be particularly important during the slow phase when voltage-gated Ca^{2+} influx is reduced. Our laboratory has observed cation channel activation subsequent to mitochondrial Ca^{2+} release (Hickey, Geiger & Magoski unpublished observation), the latter of which appears to be critical for CICR. Thus, in bag cell neurons, the activation of depolarizing conductances may rely on CICR, just as the activation of Cl^- channels does in chick DRG neurons (Ivanenko *et al*. 1993). Similarly, in rat hippocampal neurons, dantrolene application, which blocks RyRs, prevented depolarizations mediated by Ca^{2+}-activated cation channels (Partridge & Valenzuela, 1999).

Finally, several forms of Ca^{2+}-dependent plasticity, which occur during the afterdischarge, may be influenced by CICR. Such changes include the induction of the refractory period (Kaczmarek *et al*. 1982; Kaczmarek & Kauer, 1983; Magoski *et al*. 2000) and enhanced synthesis of ELH from its mRNA (Berry & Arch, 1981). These events may be mediated by CaM-kinase activity, which appropriately increases during the afterdischarge (DeRiemer *et al*. 1984). CICR and plasticity is seen in other systems; for example, in *Hermissenda* photoreceptors, changes in excitability and synaptic terminal morphology mediated through conditioning are RyR-dependent (Blackwell &

Alkon 1999; Kawai *et al*. 2004). In hippocampal dendrites, CICR triggered by Ca^{2+} influx through NMDA receptors, has a role in the induction of some forms of long-term potentiation and depression (Harvey & Collingridge, 1992; Reyes & Stanton, 1996; Emptage *et al*. 1999). Thus, mitochondrial/ER-dependent CICR in bag cell neurons may augment neurosecretion, enhance the depolarization which permits secretion, or induce several forms of Ca^{2+}-dependent plasticity.

Future Directions

Given that Ca^{2+} efflux from the ER is further regulated by IP_3 receptors, it would be interesting to examine IP_3-mediated Ca^{2+} release and how that relates to CICR in bag cell neurons. Accordingly, Ca^{2+} levels could be monitored following IP_3 injection and subsequent delivery of the afterdischarge-like train. While mitochondrial signalling that is driven by IP_3 receptors has been described previously (Pralong *et al*. 1994; Hajnoczky *et al*. 1995), experiments with either FCCP or TPP might also reveal whether mitochondrial Ca^{2+} contributes to IP_3-mediated Ca^{2+} release in bag cell neurons.

Since simulation of the bag cell neurons following dual treatment with TPP and PMA resulted in particularly broad action potentials, the effect that these agents in combination confer on CICR could not be examined in the present study. However, use of flash photolysis of Ca^{2+} to evoke CICR, would allow for TPP and PMA to be tested together without the need for delivering action potentials.

A final alteration to the stimulus protocol may also reveal if CICR truly occurs during the slow phase of stimulation. In this instance, the afterdischarge-like train would be delivered from -40 or -30 mV rather than -60 mV. Perhaps the change in membrane

potential would alter the Ca^{2+} influx and yield a component of Ca^{2+} elevation that is sensitive to TPP.

Literature Cited

Abas L, Bogoyevitch MA & Guppy M (2000). Mitochondrial ATP production is necessary for activation of the extracellular-signal-regulated kinases during ischaemia/reperfusion in rat myocyte-derived H9c2 cells. *Biochem. J.* **349**, 119-126.

Aiuchi T, Matsunaga M, Nakaya K & Nakamura Y (1985). Effects of probes of membrane potential on metabolism in synaptosomes. *Biochimica et Biophysica Acta* 843, 20-24.

Akaike N, Shirasaki T & Yakushiji T (1991). Quinolones and fenbufen interact with $GABA_A$ receptors in dissociated hippocampal cells of rat. *J Neurophys* **66**, 497-504.

Akerman K (1978). Effect of pH and Ca^{2+} on the retention of Ca^{2+} uptake in rat liver mitochondria. *Arch Biochem Biophys* **189**, 256-262.

Albert AP & Large WA (2002). A Ca^{2+}-permeable non-selective cation channel activated by depletion of internal Ca^{2+} stores in single rabbit portal vein myocytes. *J Physiol* **538**, 717-728.

Allemann N & Schneider A (2000). ATP production in isolated mitochondria of procyclic *Trypanosoma brucei*. *Mol Biochem Parasitol* **111**, 87-94.

Arch S (1972a). Polypeptide secretion from the isolated parietovisceral ganglion of *Aplysia californica*. *J Gen Physiol* **59**, 47-59.

Arch S (1972b). Biosynthesis of the egg-laying hormone (ELH) in the bag cell neurons of *Aplysia californica*. *J Gen Physiol* **60**, 102-119.

Armstrong WE, Smith BN, & Tian M (1994). Electrophysiological characteristics of immunochemically identified rat oxytocin and vasopressin neurones in vitro. *J Physiol* **475**, 115–128.

Arnaudeau S, Kelley W, Walsh J, & Demaurex N (2001). Mitochondria recycle Ca^{2+} to the endoplasmic reticulum and prevent the depletion of neighboring endoplasmic reticulum regions, *J. Biol. Chem* **276**, 29430-29439.

Ashcroft FM & Rorsman P (1989). Electrophysiology of the pancreatic β-cell. *Prog Biophys Mol Biol* **54**, 87-143.

Augustine GJ, Santamaria F & Tanaka K (2003). Local calcium signaling in neurons *Neuron* **40**, 331-346.

Bardo S, Cavazzini MG & Emptage N (2006). The role of the endoplasmic reticulum Ca^{2+} store in the plasticity of central neurons. *Trends Pharmacol Sci* **27**, 78-84.

Baksh S & Michalak M (1991). Expression of calreticulin in *Escherichia coli* and identification of its Ca^{2+} binding domains. *J Biol Chem*. **266**, 21458-21465.

Bean BP (1989). Classes of calcium channels in vertebrate cells. *Annu Rev Physiol* **51**, 367-384.

Beaumont V, Llobet A & Lagnado L (2005). Expansion of calcium microdomains regulates fast exocytosis at a ribbon synapse. *Proc Natl Acad Sci* **102**, 10700-10705.

Beck A, Lohr C & Deitmer JW (2001). Calcium transients in subcompartments of the leech Retzius neuron as induced by single action potentials. *J Neurobiol* **48**, 1-18.

Berridge MJ (1998). Neuronal calcium signalling. *Neuron* **21**, 13-26.

Berry RW & Arch S (1981). Activation of neurosecretory cells enhances their synthesis of secretory protein. *Brain Res* **215**, 115-123.

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

Blackwell KT & Alkon DL (1999). Ryanodine receptor modulation of in vitro associative learning in *Hermissenda crassicornis*. *Brain Res* **822**, 114-125.

Blankenship JE & Haskins JT (1979). Electrotonic coupling among neuroendocrine cells in *Aplysia*. *J Neurophys* **42**, 347-355.

Borst JGG & Sakmann B (1996). Calcium influx and transmitter release in a fast CNS synapse. *Nature* **383**, 431-434.

Bouron A (2000). Activation of a capacitative Ca^{2+} entry pathway by store depletion in cultured hippocampal neurones. *FEBS Lett*. **470**, 269-272.

Byrne JH & Kandel ER (1996). Presynaptic facilitation revisited: state and time dependence. *J. Neurosci* **16**, 425-435.

Carafoli E (1979). Intracellular calcium homeostasis. *Annual Review of Biochemistry* **56**, 395-433.

Carbone E & Swandulla D (1989). Neuronal calcium channels: kinetics, blockade and modulation. *Prog Biophys Mol Biol* **54**, 31-58.

Castagna M, Takay Y, Karibuchi K, Sano K, Kikkawa U & Nishizuka Y (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor promoting phorbol esters. *J Biol Chem* **257**, 7847-7851.

Casteels R & Droogmans G (1981). Exchange characteristics of the noradrenaline-sensitive calcium store in vascular smooth muscle cells of rabbit ear artery. *J Physiol* **317**, 263-279.

Chameau P, Van de Vrede Y, Fossier P & Baux G (2001). Ryanodine-, IP_3- and NADP-dependent calcium stores control acetylcholine release. *Pflugers Arch* **443**, 289-296.

Chiu AY, Hunkapiller MW, Heller E, Stuart DK, Hood LE & Strumwasser F (1979). Purification and primary structure of the neuropeptide egg-laying hormone of *Aplysia californica*. *Proc Natl Acad Sci* **76**, 6656-6660.

Clapham DE (1995). Calcium signaling. *Cell* **80**, 259-268.

Clementi E, Racchetti G, Zacchetti D, Panzeri MC & Meldolesi J (1992). Differential expression of markers and activities in a group of PC12 nerve cell clones. *Eur J Neurosci* **4**, 944-953.

Cohen AS, Moore KA, Bangalore R, Jafri MS, Weinreich D & Kao JP (1997). Ca^{2+}-induced Ca^{2+} release mediates Ca^{2+} transients evoked by single action potentials in rabbit vagal afferent neurones. *J Physiol* **499**, 315–328.

Colegrove SL, Albrecht MA & Friel DD (2000a). Dissection of mitochondrial Ca^{2+} uptake and release fluxes in situ after depolarization evoked $[Ca^{2+}]_i$ elevations in sympathetic neurons. *J Gen Physiol* **115**, 351-369.

Colegrove SL, Albrecht MA & Friel, DD (2000b). Quantitative analysis of mitochondrial Ca^{2+} uptake and release pathways in sympathetic neurons: reconstruction of the recovery after depolarization-evoked $[Ca^{2+}]_i$ elevations. *J Gen Physiol* **115**, 371-388.

Collins TJ, Lipp P, Berridge MJ, Li W& Bootman MD (2000). Inositol 1,4,5-trisphosphate-induced Ca^{2+} release is inhibited by mitochondrial depolarization. *Biochem J* **347**, 593-600.

Conn PJ, Strong JA, Azhderian EM, Nairn AC, Greengard P & Kaczmarek LK (1989a). Protein kinase inhibitors selectively block phorbol ester- or forskolin-induced changes in excitability of *Aplysia*. *J Neurosci* **9**, 473-479.

Conn PJ, Strong JA & Kaczmarek LK (1989b). Inhibitors of protein kinase C prevent enhancement of calcium current and action potentials in peptidergic neurons of *Aplysia*. *J Neurosci* **9**, 480-487.

Conn PJ & Kaczmarek LK (1989). The bag cell neurons of *Aplysia*. *Mol Neurobiol* **3**, 237-273.

Cruz LJ, Corpus G, Olivera BM (1976). A preliminary study of *Conus* venom protein. *Veliger* **18**, 302-308.

Curtis TM & Scholfield CN (2001). Nifedipine blocks Ca^{2+} store refilling through a pathway not involving L-type Ca^{2+} channels in rabbit arteriolar smooth muscle. *J Physiol* **532**, 609-623.

DeRiemer SA, Kaczmarek LK, Lai Y, McGuiness TL & Greengard P (1984). Calcium/calmodulin-dependent protein phosphorylation in the nervous system of *Aplysia*. *J Neurosci* **4**, 1618-1625.

DeRiemer SA, Strong JA, Albert KA, Greengard P & Kaczmarek LK (1985). Enhancement of calcium current in *Aplysia* neurones by phorbol ester and protein kinase C. *Nature* **313,** 313-6.

Duan K, Yu X, Zhang C & Zhoe Z (2003). Control of secretion by temporal patterns of action potentials in adrenal chromaffin cells. *J Neurosci* **23**, 11235-11243.

Dudek FE, Cobbs JS & Pinsker HM (1979). Bag cell electrical activity underlying spontaneous egg laying in freely behaving *Aplysia brasiliana*. *J Neurophysiol* **42**, 804-817.

Dudek FE & Kossatz A (1982). Conduction velocity and spike duration during afterdischarge in neuroendocrine bag cells of *Aplysia*. *J Neurobiol* **13**, 319-326.

Durante P, Cardenas CG, Whittaker JA, Kitai ST & Scroggs RS (2004). Low-threshold L-type calcium channels in rat dopamine neurons. *J Neurophysiol* **91**, 1450-1454.

Eliot LS, Kandel ER, Siegelbaum SA & Blumenfeld H (1993). Imaging terminals of *Aplysia* sensory neurons demonstrates role of enhanced Ca^{2+} influx in presynaptic facilitation Nature 361, 634 –637.

Emptage N, Bliss TVP & Fine A (1999). Single synaptic events evoke NMDA receptor-mediated release of calcium from internal stores in hippocampal dendritic spines *Neuron* **22**, 115-124.

Endo M, Tanaka M & Ogawa Y (1970). Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. *Nature* **228**, 34-36.

Ertel EA, Campbell KP, Harpold MM, Hofmann F, Mori Y, Perez-Reyes E, Schwartz A, Snutch TP, Tanabe T & Birnbaumer L (2000). Nomenclature of voltage-gated calcium channels. *Neuron* **25**, 533-535.

Fabiato A & Fabiato F (1975). Contractions induced by calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. *J Physiol* **249**, 469-495.

Fatt P & Katz B (1953). The electrical properties of crustacean muscle fibres. *J Physiol* **130**, 171-204.

Fine J, Cole P, & Davidson JS (1989). Extracellular nucleotides stimulate receptor-mediated calcium mobilization and inositol phosphate production in human fibroblasts. *Biochem J*. **263**, 371–376.

Fink LA, Connor JA & Kaczmarek LK (1988). Inositol triphosphate releases intracellularly stored calcium and modulates ion channels in molluscan neurons. *J Neurosci* **8**, 2544–2555.

Fisher T, Levy S & Kaczmarek LK (1994). Transient changes in intracellular calcium associated with a prolonged increase in excitability in neurons of *Aplysia californica*. *J Neurophysiol* **71**, 1254-1257.

Franklin I, Winz R & Hubbard M (2001). Endoplasmic reticulum Ca^{2+}-ATPase pump is up-regulated in calcium-transporting dental enamel cells: a non-housekeeping role for SERCA2b. *Biochem J* **358**, 217-224.

Friel DD & Tsien RW (1992). A caffeine- and ryanodine-sensitive Ca^{2+} store in bullfrog sympathetic neurons modulates effects of Ca^{2+} entry on $[Ca^{2+}]_i$. *J Physiol* **450**, 217-246.

Friel DD & Tsien RW (1994). An FCCP-sensitive Ca^{2+} store in bullfrog sympathetic neurons and its participation in stimulus-evoked changes in $[Ca^{2+}]_i$. *J Neurosci* **14**, 4007-4024.

Gafni J, Munsch J, Lam T, Catlin M, Costa L, Molinski T & Pessah I (1997). Xestospongins: Potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. *Neuron* **19**, 723-733.

Gilabert JA & Parekh AB (2000). Respiring mitochondria determine the pattern of activation and inactivation of the store-operated Ca^{2+} current I_{CRAC}. *EMBO J* **19**, 6401-6407.

Giovannucci DR, Hlubek MD & Stuenkel EL (1999). Mitochondria regulate the Ca^{2+}-exocytosis relationship of bovine adrenal chromaffin cells. *J Neurosci* **19**, 9261-9270.

Gorman AL & Thomas MV (1980). Intracellular calcium accumulation during depolarization in a molluscan neurone. *J Physiol* **308**, 259-285.

Gunter KK & Gunter TE (1994). Transport of calcium by mitochondria. *J Bioenerg Biomembr* **26**, 471- 485.

Gunter TE & Pfeiffer DR (1990). Mechanisms by which mitochondria transport calcium. *Am J Physiol* **258,** 755-786.

Grynkiewicz G, Poenie M & Tsien RY (1985). A new generation of calcium indicators with greatly improved fluorescent properties. *J Biol Chem* **260**, 3440-3448.

Hagiwara, S (1983). *Membrane Potential-Dependent Ion Channels in Cell Membrane. Phylogenetic and Developmental Approaches.* Raven Press, New York.

Hajnoczky G, Robb-Gaspers LD, Seitz MB & Thomas AP (1995). Decoding of cytosolic calcium oscillations in the mitochondria. *Cell* **82**, 415-424.

Hallam TJ & Rink TJ (1985). Agonists stimulate divalent cation channels in the plasma membrane of human platelets. *FEBS Lett*. **186**, 175-179.

Hardingham G & Bading H (1998). Nuclear calcium: a key regulator of gene expression *BioMetals* **11**, 345–358.

Harvey J & Collingridge GL (1992). Thapsigargin blocks the induction of long-term potentiation in rat hippocampal slices. *Neuroscience Letters* **139**, 197-200.

Heytler PG & Prichard WW (1962). A new class of uncoupling agents-carbonyl cyanide phenylhydrazones. *Biochem Biophys Res Commun* **7**, 272-275.

Hoth M & Penner R (1993). Calcium release-activated calcium current in rat mast cells. *J Physiol* **465**, 359-386.

Huguenard JR (1996). Low-threshold calcium currents in central nervous system neurons. *Annu Rev Physiol* **58** 329-348.

Hille B (2003). In: *Ionic channels of excitable membranes.* Sinauer Associates, USA

Hua SY, Nohmi M & Kuba K (1993). Characteristics of Ca^{2+} release induced by Ca^{2+} influx in cultured bullfrog sympathetic neurons. *J Physiol* **464**, 245-272.

Hung AY & Magoski NS (2007). Activity-dependent initiation of a prolonged depolarization in *Aplysia* bag cell neurons: role for a cation channel. *J Neurophysiol* **97**, 2465-2479.

Imagawa T, Smith JS, Coronado R & Campbell KP (1987). Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca^{2+}-permeable pore of the calcium release channel. *J Biol Chem* **262**, 16636–16643.

Ishii K, Hirose K & Iino M (2006). Ca^{2+} shuttling between endoplasmic reticulum and mitochondria underlying Ca^{2+} oscillations. *EMBO Rep* **7**, 390-396.

Ivanenko A, Baring MD, Airey JA, Sutko JL & Kenyon JL (1993). A caffeine- and ryanodine-sensitive Ca^{2+} store in avian sensory neurons. *J Neurophys* **70**, 710-722.

Jackson TR, Patterson SI, Thastrup O & Hanley MR (1988). A novel tumour promoter, thapsigargin, transiently increases cytoplasmic free Ca^{2+} without generation of inositol phosphates in NG115-401L neuronal cells. *Biochem J* **253**, 81-86.

Jackson JG & Thayer SA (2006). Mitochondrial modulation of Ca^{2+}-induced Ca^{2+}-release in rat sensory neurons. *J Neurophysiol* **96**, 1093-1104.

Johnson LV, Walsh ML, Bockus BJ & Chen LB (1981). Monitoring of relative mitochondrial membrane potential in living cells by fluorescene microscopy. *J Cell Biol* **88**, 526-535.

Jonas E, Knox R, Smith T, Wayne N, Connor J & Kaczmarek L (1997). Regulation by insulin of a unique neuronal Ca^{2+} pool and neuropeptide secretion. *Nature* **385,** 343-346.

Kachoei B, Imperial JS, Olivera BM & Magoski NS (2004). Actions of *Conus textile* venom on *Aplysia* bag cell neurons. *Soc Neurosci Abstr* 30:86.2.

Kachoei BA, Knox RJ, Uthuza D, Levy S, Kaczmarek LK & Magoski NS (2006). A store-operated Ca^{2+} influx pathway in the bag cell neurons of *Aplysia*. *J Neurophysiol* **96**, 2688-2698.

Kaczmarek LK, Jennings K & Strumwasser F (1982). An early sodium and a late calcium phase in the afterdischarge of peptide-secreting neurons of *Aplysia*. *Brain Res* **238**, 105-115.

Kaczmarek LK & Kauer JA (1983). Calcium entry causes a prolonged refractory period in peptidergic neurons of *Aplysia*. *J Neurosci* **3**, 2230-2239.

Kaczmarek LK & Strumwasser F (1984). A voltage-clamp analysis of currents in isolated peptidergic neurons of *Aplysia*. *J Neurophysiol* **52**, 340-349.

Kandel ER (1976). *Cellular Basis of Behavior.* WH Freeman and Company, USA.

Kang G & Holz GG (2003). Amplification of exocytosis by Ca^{2+}-induced Ca^{2+} release in INS-1 pancreatic ß cells. *J Physiol* **546**, 175-189.

Karadjov JS, Kudzina LY & Zinchenko VP (1986). TPP^{+} inhibits Na^{+}-stimulated Ca^{2+} efflux from brain mitochondria. *Cell Calcium* **7**, 115-119.

Kawai R, Horikoshi T & Sakakibara M (2004). Involvement of the ryanodine receptor in morphologic modification of *Hermissenda* type B photoreceptors after *in vitro* conditioning. *J Neurophysiol* **91**, 728–735.

Kim MH, Korogod N, Schneggenburger R, Ho WK & Lee SH (2005). Interplay between Na^{+}/Ca^{2+} exchangers and mitochondria in Ca^{2+} clearance at the Calyx of Held *J Neuroscience* **25**, 6057-6065.

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

Klein M & Kandel ER (1978). Presynaptic modulation of voltage-dependent Ca^{2+} current: mechanism for behavioral sensitization in *Aplysia californica. Proc Natl Acad Sci* **75**, 3512-3516.

Knox RJ, Jonas EA, Kao L-S, Smith PJS, Connor JA, & Kaczmarek LK (1996). Ca^{2+} influx and activation of a cation current are coupled to intracellular Ca^{2+} release in peptidergic neurons of *Aplysia californica. J Physiol* **494**, 27-693.

Kostyuk PG (1989). Diversity of calcium ion channels in cellular membranes. *Neuroscience* **28**, 253-261.

Kostyuk PG & Verkhratsky A (1994). Calcium stores in neurons and glia. *Neuroscience* **63**, 381-404.

Kupfermann I (1967). Stimulation of egg laying: possible neuroendocrine function of bag cells of abdominal ganglion of *Aplysia californica. Nature* **216**, 814-815.

Kupfermann I & Kandel ER (1970). Electrophysiological properties and functional interconnections of two symmetrical neurosecretory clusters (bag cells) in abdominal ganglion of *Aplysia. J Neurophysiol* **33**, 865-876.

Lehninger AL, Carafoli E & Rossi CS (1967). Energy linked ion movements in mitochondrial systems. *Adv Enzymol* **29**, 259-320.

Levitan IB & Kaczmarek LK (2002). The neuron: cell and molecular biology. Oxford Univ. Press, New York.

Lipscombe D, Madisona DV, Poenieb M, Reutera H, Tsiena RW & Tsien RY (1988). Imaging of cytosolic Ca^{2+} transients arising from Ca^{2+} stores and Ca^{2+} channels in sympathetic neurons. *Neuron* **1**, 355-365.

Liu YJ & Gylfe E (1997). Store-operated Ca^{2+} entry in insulin-releasing pancreatic ß-cells. *Cell Calcium* **22**, 277-286.

Llinas R, Sugimori M, Lin J-W & Cherksey B (1989). Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FIX) from funnel-web spider poison. *Proc Natl Acad Sci (USA)* **86**, 1689-1693.

Llinas R, Sugimori M & Silver RB (1992). Microdomains of high calcium concentrations in a presynaptic terminal. *Science* **256**, 677-679.

Loechner KJ, Azhderian EM, Dreyer R,& Kaczmarek LK (1990). Progressive potentiation of peptide release during a neuronal discharge. *J Neurophysiol* **63**, 738-744.

Lupinsky DA & Magoski NS (2006). Ca^{2+}-dependent regulation of a non-selective cation channel from *Aplysia* bag cell neurones. *J Physiol* **575**, 491-506.

Magoski NS, Knox RJ & Kaczmarek LK (2000). Activation of a Ca^{2+}-permeable cation channel produces a prolonged attenuation of intracellular Ca^{2+} release in *Aplysia* bag cell neurones. *J Physiol* **522**, 271-283.

Magoski NS, Wilson GF & Kaczmarek LK (2002). Protein kinase modulation of a neuronal cation channel requires protein-protein interactions mediated by an Src homology 3 domain. *J Neurosci* **22**, 1–9.

Magoski NS & Geiger JE (2006). Ca^{2+} entry through a non-selective cation channel in *Aplysia* bag cell neurons. *Soc Neurosci Abstr* 32:813.7

Magoski NS & Kaczmarek LK (2005). Association/Dissociation of a channel-kinase complex underlies state-dependent modulation. *J Neurosci* **25**, 8037-8047.

Malli R, Frieden M, Osibow K, Zoratti C, Mayer M, Demaurex N, & Graier W (2003). Sustained Ca^{2+} Transfer across Mitochondria Is Essential for Mitochondrial Ca^{2+} Buffering, Store-operated Ca^{2+} Entry, and Ca^{2+} Store Refilling. *J. Biol. Chem* **278**, 44769-44779.

Marhl M, Haberichter T, Brumen M & Heinrich R (2000). Complex calcium oscillations and the role of mitochondria and cytosolic proteins. Biosystems **57**, 75-86.

Matlib MA, Zhou Z, Ahmed KS, Choi KM, Krause-Baueri J, Phillips R, Altschuld R, Katsube Y, Sperelakis N & Bers DM (1998). Oxygen-bridged dinuclear ruthenium amine complex specifically inhibits Ca^{2+} uptake into mitochondria in vitro and in situ in single cardiac myocytes. *J Biol Chem* **273**, 10223-10231.

Mayeri E, Brownell P, Branton WD & Simon SB (1979a). Multiple, prolonged actions of neuroendocrine bag cells on neurons in *Aplysia*. I. Effects on bursting pacemaker neurons. *J Neurophysiol* **42**, 1165-1184.

Mayeri E, Brownell P & Branton WD (1979b). Multiple, prolonged actions of neuroendocrine bag cells on neurons in *Aplysia.* II. Effects on beating pacemaker and silent neurons. *J Neurophysiol* **42**, 1185-1197.

Meissner G (1986). Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. *J. Biol. Chem* **261**, 6300-6306.

Meldolesi J (2001). Rapidly exchanging Ca^{2+} stores in neurons: molecular, structural and functional properties. *Prog Neurobiol* **65**, 309-338.

Michel S & Wayne NL (2002). Neurohormone secretion persists after post afterdischarge membrane depolarization and cytosolic calcium elevation in peptidergic neurons in intact nervous tissue. *J Neurosci* **22**, 9063-9069.

Miyata H, Silverman HS, Sollott SJ, Lakatta EG, Stern MD & Hansford RG (1991). Measurement of mitochondrial free Ca^{2+} concentration in living single rat cardiac myocytes. *Am J Physiol* **261**, H1123–H1134.

Mothet JP, Fossier P, Meunier FM, Stinnakre J, Tauc L & Baux G (1998). Cyclic ADP-ribose and calcium-induced calcium release regulate neurotransmitter release at a cholinergic synapse of *Aplysia. J Physiol* **507**, 405-414.

Mulle C, Choquet D, Kern H, & Changeux JP (1992). Calcium influx through nicotinic receptor in rat central neurons: its relevance to cellular regulation. *Neuron* **8**,135-143.

Murayama T, Kurebayashi N & Ogawa Y (2000). Role of Mg^{2+} in Ca^{2+}-induced Ca^{2+} release through ryanodine receptors of frog skeletal muscle: modulations by adenine nucleotides and caffeine. *Biophys J* **78**, 1810–1824.

Neering IR & McBurney RN (1984). Role for microsomal Ca^{2+} storage in mammalian neurones? *Nature* **309**, 158-160.

Nelson TJ, Zhao W-Q, Yuan S, Favit A, Pozzo-Miller L & Alkon DH (1999). Calexcitin interaction with neuronal ryanodine receptors. *Biochem J* **341**, 423–433.

Nelson DL & Cox MM (2000). *Lehninger principles of biochemistry*. Worth, New York, USA

Nichols R & Mollard P (1996). Direct observation of serotonin 5-HT3 receptor-induced increases in calcium levels in individual brain nerve terminals. *J Neurochemistry* **67**, 581 -592.

Nilius B (2003). From TRPs to SOCs, CCEs, and CRACs: consensus and controversies. *Cell Calcium* **33**, 293-298.

Nowycky M, Fox AP & Tsien RW (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* **316**, 440 – 443.

Orkand RK & Thomas RC (1995). Effects of low doses of caffeine on $[Ca^{2+}]_i$ in voltage-clamped snail (*Helix aspersa*) neurones. *J Physiol* **489**, 19-28.

Ouardouz M, Nikolaeva MA, Coderre E, Zamponi GW, McRory JE, Trapp BD, Yin X, Wang W, Woulfe J, & Stys PK (2003). Depolarization-induced Ca^{2+} release in ischemic spinal cord white matter involves L-type Ca^{2+} channel activation of ryanodine receptors. *Neuron* **40**, 53–63.

Park D & Dunlap K (1998). Dynamic regulation of calcium influx by G-proteins, action potential waveform, and neuronal firing frequency. *J Neurosci* **18**, 6757-6766.

Parekh AB (2003). Store-operated Ca^{2+} entry: dynamic interplay between endoplasmic reticulum, mitochondria and plasma membrane. *J Physiol* **547**, 333-348.

Partridge LD & Valenzuela CF (1999). Ca^{2+} stores-dependent potentiation of Ca^{2+}-activated non-selective cation channels in rat hippocampal neurones in vitro. *J Physiol* **521**, 617-627.

Pauceka P & Jabůrekb M (2004). Kinetics and ion specificity of Na^{+}/Ca^{2+} exchange mediated by the reconstituted beef heart mitochondrial Na^{+}/Ca^{2+} antiporter *Biochim Biophys Acta* **1659**, 83-91.

Peterson BZ, Lee JS, Mulle JG, Wang Y, Leon M, & Yue DT (2000). Critical determinants of Ca21-dependent inactivation within an EF-hand motif of L-type Ca^{2+} channels. *Biophys J* **78**, 1906-1920.

Plant TD & Schaefer M (2003). Receptor-operated Ca^{2+} permeable non-selective cation channels. *Cell Calcium* **33**,441-450.

Pinsker H & Dudek F (1977). Bag cell control of egg laying in freely behaving *Aplysia*. *Science* **197**, 490-493.

Piper DR & Lucero MT (1999). Calcium signalling in squid olfactory receptor neurons. *Biol Sig Rec* **8**, 329-337.

Powis DA, Clark CL, & O'Brien KJ (1996). Depeted internal store-activated Ca^{2+} entry can trigger neurotransmitter release in bovine chromaffin cells. *Neurosci Lett* **204**, 165-168.

Pozzan T, Rizzuto R, Volpe P & Meldolesi J (1994). Molecular and cellular physiology of intracellular Ca^{2+} stores. *Physiol Rev* **74**, 595–636.

Prakriya M & Lewis RS (2003). CRAC channels: activation, permeation, and the search for a molecular identity. *Cell Calcium* **33**, 311-321.

Pralong W-F, Spät A & Wollheim CB (1994). Dynamic pacing of cell metabolism by intracellular Ca^{2+}. *J Biol Chem* **269**, 27310–27314.

Prothero LS, Mathie A, & Richards CD (2000). Purinergic and muscarinic receptor activation activates a common calcium entry pathway in rat neocortical neurons and glial cells. *Neuropharmacol* **39**, 1768-1778.

Purves D, Augustine GJ, Fitzpatrick D, Katz LC, LaMantia A-S, McNamara JO, Williams SM (2001). In: *Neuroscience*. Sinauer Associates, USA

Putney J (1986). A model for receptor-regulated calcium entry. *Cell Calcium* **7**, l-12.

Puskin JS, Gunter TE, Gunter KK & Russell PR (1976). Evidence for more than one Ca^{2+} transport mechanism in mitochondria. *Biochemistry* **15**, 3834-3842.

Quattrocki EA, Marshall J & Kaczmarek LK (1994). A Shab potassium channel contributes to action potential broadening in peptidergic neurons. *Neuron* **12**, 73-86.

Reyes M & Stanton PK (1996). Induction of hippocampal long-term depression requires release of Ca^{2+} from separate presynaptic and postsynaptic intracellular stoves. *J Neurosci* **16**, 5951-5960.

Richter TA, Kolaj M & Renaud LP (2005). Low voltage-activated Ca^{2+} channels are coupled to Ca^{2+}-induced Ca^{2+} release in rat thalamic midline neurons. *J Neurosci* **25**, 8267-8271.

Ringer S (1883). A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. *J Physiol*. **4**, 29-42.

Rodericka H, Lechleiterb J, & Camachoa P (2000). Cytosolic Phosphorylation of Calnexin Controls Intracellular Ca^{2+} Oscillations via an Interaction with SERCA2b. *The Journal of Cell Biology* **149**, 1235-1248.

Rodney G, Wilson G, & Schneider M (2005). A Calmodulin Binding Domain of RyR Increases Activation of Spontaneous Ca^{2+} Sparks in Frog Skeletal Muscle. *The Journal of biological chemistry* **280**, 11713–11722.

Rothman BS, Weir G, & Dudek FE (1988). Egg-laying hormone: direct action on the ovotestis of *Aplysia. Gen Comp Endocrinol* **52**, 134-141.

Rousseau E, Ladine J, Liu QY & Meissner G (1988). Activation of the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum by caffeine and related compounds. *Arch Biochem Biophysics* **267**, 75-86.

Sage SO, Merritt JE, Hallam TJ & Rink TJ (1989). Receptor mediated calcium entry in fura-2-loaded human platelets stimulated with ADP and thrombin. *Biochem J* **258**, 923-926.

Scheller RH, Jackson JF, McAllister LB, Rothman BS, Mayeri E & Axel R (1983). A single gene encodes multiple neuropeptides mediating a stereotyped behavior. *Cell* **32**, 7-22.

Shmigol A, Kostyuk P & Verkhratsky A (1995). Dual action of thapsigargin on calcium mobilization in sensory neurons: inhibition of Ca^{2+} uptake by caffeine-sensitive pools and blockade of plasmalemmal Ca^{2+} channels. *Neuroscience* **65**, 1109–1118.

Seidler NW, Jona I, Vegh M & Martonosi A (1989). Cyclopiazonic acid is a specifiec inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J Biol Chem* **264**, 17816-17823.

Simon MI, Strathmann MP, & Gautam N (1991). Diversity of G proteins in signal transduction. *Science* **252**, 802-808.

Simpson PB & Russell JT (1996). Mitochondria support inositol 1,4,5-trisphosphate-mediated Ca^{2+} waves in cultured oligodendrocytes. *J Biol Chem* **271**, 33493-33501.

Smith SJ, MacDermott AB & Weight FF (1983). Detection of intracellular Ca^{2+} transients in sympathetic neurones using arsenazo III. *Nature* **304**, 350-352.

Solovyova N, Veselovsky N, Toescu EC & Verkhratsky A (2002). Ca^{2+} dynamics in the lumen of the endoplasmic reticulum in sensory neurons: direct visualization of Ca^{2+}-induced Ca^{2+} release triggered by physiological Ca^{2+} entry. *EMBO J* **21**, 622-630.

Sossin W & Schwartz JH (1994). Translocation of protein kinase Cs in *Aplysia* neurons: evidence for complex regulation. *Mol Brain Res* **24**, 210-218.

Stauderman KA & Pruss RM (1989). Dissociation of Ca^{2+} entry and Ca^{2+} mobilization responses to angiotensin II in bovine adrenal chromaffin cells. *J Biol Chem* **264**, 18349-18355.

Strong JA, Fox AP, Tsien RW & Kaczmarek LW (1987). Stimulation of protein kinase C recruits covert calcium channels in *Aplysia* bag cell neurons. *Nature* **325**, 714-717.

Stuart DK, Chiu AY & Strumwasser F (1980). Neurosecretion of egg-laying hormone and other peptides from electrically active bag cell neurons of *Aplysia*. *J Neurophysiol* **43**, 488-498.

Stuart DK & Strumwasser F (1980). Neuronal sites of action of a neurosecretory peptide, egg-laying hormone, in *Aplysia californica. J. Neurophysiol* **43**, 499-519.

Su Z, Csutora P, Hunton D, Shoemaker RL, Marchase RB & Blalock JE (2001). A store-operated nonselective cation channel in lymphocytes is activated directly by Ca^{2+} influx factor and diacylglycerol. *Am J Physiol Cell Physiol* **280**, C1284–C1292.

Suryanarayanan A & Slaughter MM (2006). Synaptic transmission mediated by internal calcium stores in rod photoreceptors. *J Neurosci* **26**, 1759-1766.

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

Thomas J, Masgrau R, Churchill G and Galione A (2001). Pharmacological characterization of the putative cADP-ribose receptor. *Biochem. J* **359**, 451–457.

Tillotson D (1979). Inactivation of Ca^{2+} conductance dependent on entry of Ca^{2+} ions in molluscan neurons. *Proc Natl Acad Sci* **76**, 1497-1500.

Trepakova ES, Gericke M, Hirakawa Y, Weisbrod RM, Cohen RA, & Bolotina VM (2001). Properties of a native cation channel activated by Ca^{2+} store depletion in vascular smooth muscle cells. *J Biol Chem* **276**, 7782-7790.

Trueta C, Sánchez-Armass S, Morales MA & De-Miguel FF (2004). Calcium-induced calcium release contributes to somatic secretion of serotonin in leech Retzius neurons. *J Neurobiol* **61**, 309-316.

Tsien RW, Lipscombe D, Madison DV, Bley KR & Fox P (1988). Multiple types of neuronal calcium channels and their selective modulation. *Trends NeuroSci* **11**, 431-438.

Tu H, Nelson O, Bezprozvanny A, Wang Z, Lee S-F, Hao Y-H, Serneels L, Strooper BD, Yu G & Bezprozvanny I (2006). Presenilins form ER Ca^{2+} leak channels, a function disrupted by Familial Alzheimer's Disease-linked mutations. *Cell* **126**, 981-993.

Usachev YM & Thayer SA (1997). All-or-none Ca^{2+} release from intracellular stores triggered by Ca^{2+} influx through voltage-gated Ca^{2+} channels in rat sensory neurons. *J Neurosci* **17**, 7404-7414.

Usachev YM, Marsh AJ, Johanns TM, Lemke MM & Thayer SA (2006). Activation of protein kinase C in sensory neurons accelerates Ca^{2+} uptake into the endoplasmic reticulum. *J Neurosci* **26**, 311-318.

Verkhratsky A (2005). Physiology and pathophysiology of the calcium store. *Physiol Rev* **85**, 201-279.

Villalobos C & Garcia-Sancho J (1995). Capacitative Ca^{2+} entry contributes to the Ca^{2+} influx induced by thyrotropin-releasing hormone (TRH) in GH3 pituitary cells. *Eur J Physiol* **430**, 923-935.

Vorndran C, Minta A & Poenie M (1995). New fluorescent calcium indicators designed for cytosolic retention or measuring calcium near membranes. *Biophys J* **69**, 2112-2124.

Vu CQ, Lu P-J, Chen C-S & Jacobson MK (1996). 2`-Phospho-cyclic ADP-ribose, a calcium-mobilizing agent derived from NADP. *J Biol Chem* **271**, 4747-4754.

Wayne NL, Kim J, & Lee E (1998a). Prolonged hormone secretion from neuroendocrine cells of Aplysia is independent of extracellular calcium. *J Neuroendocrinol* **10**, 529-537.

Wayne NL, Lee W & Kim YJ (1999). Persistent activation of calcium-activated and calcium-independent protein kinase C in response to electrical afterdischarge from peptidergic neurons of *Aplysia*. *Brain Res* **834**, 211-213.

Weber A (1968). The mechanism of the action of caffeine on sarcoplasmic reticulum. *J Gen Physiol* **52**, 760-772.

Werth JL & Thayer SA (1994). Mitochondria buffer physiological calcium loads in cultured rat dorsal root ganglion neurons. *J Neurosci* **14**, 348-356.

Whim MD & Kaczmarek LK (1998). Heterologous expression of the Kv3.1 potassium channel eliminates spike broadening and the induction of a depolarizing afterpotential in the peptidergic bag cell neurons. *J Neurosci* **18**, 9171-9180.

White RE & Hartzell HC (1989). Magnesium ions in cardiac function. Regulator of ion channels and second messengers. *Biochem Pharmac* **38**, 859-867.

White BH & Kaczmarek LK (1997). Identification of a vesicular pool of calcium channels in the bag cell neurons of *Aplysia california. J Neurosci* **17**, 1582-1595.

Wingrove DE & Gunter TE (1986). Kinetics of mitochondrial calcium transport II. A kinetic description of the sodium-dependent calcium efflux mechanism of liver mitochondria and inhibition by ruthenium red and by tetraphenylphosphonium. *J Biol Chem* **261**, 15166-15171.

Wilson GF & Kaczmarek LK (1993). Mode-switching of a voltage-gated cation channel is mediated by a protein kinase A-regulated tyrosine phosphatase. *Nature* **366**, 433-438.

Wilson GF, Richardson FC, Fisher TE, Olivera BM & Kaczmarek LK (1996). Identification and characterization of a Ca^{2+}-sensitive nonspecific cation channel underlying prolonged repetitive firing in *Aplysia* neurons. *J Neurosci* **16**, 3661-3671.

Yagodina S, Pivovarovab N, Andrews B & Sattelle D (1999). Functional characterization of thapsigargin and agonist-insensitive acidic Ca^{2+} stores in Drosophila melanogaster S2 cell lines, *Cell Calcium* **25**, 29-38.

Ylitalo KV, Ala-Rämia A, Liimattaa EV, Peuhkurinenb KJ & Hassinen IE (2000). Intracellular free calcium and mitochondrial membrane potential in ischemia/reperfusion and preconditioning. *J Mol Cell Cardio* **32**, 1223-1238.

Zhang JF, Randall AD, Ellinor PT, Horne WA, Sather WA, Tanabe T, Schwartz TL & Tsien RW (1993). Distinctive pharmacology and kinetics of cloned neuronal Ca^{2+} channels and their possible counterparts in mammalian neurons. *Neuropharm* **32**, 1075-1088.

Zhang Y, Magoski NS & Kaczmarek LK (2002). Prolonged activation of Ca^{2+}-activated K^+ current contributes to the long-lasting refractory period of *Aplysia* bag cell neurons. *J Neurosci* **22**, 10134-10141.

Zhong N, Beaumont V & Zucker RS (2001). Role for mitochondrial and reverse mode Na^+/Ca^{2+} exchange and the plasmalemma Ca^{2+} ATPase in post-tetanic potentiation at crayfish neuromuscular junctions*. J Neurosci* **21**, 9598-9607.

Zorzato F, Scutari E, Tegazzin V, Clementi E & Treves S (1993). Chlorocresol: an activator of ryanodine receptor-mediated Ca^{2+} release. *Mol Pharmacol* **44**, 1192-1201.

Zuhlke RD, Pitt GS, Deisseroth K, Tsien RW & Reuter H (1999). Calmodulin supports both inactivation and facilitation of L-type calcium channels. *Nature* **399**, 159-162.

Zweifach A & Lewis RS (1993). Mitogen-regulated Ca^{2+} current of T lymphocytes is activated by depletion of intracellular Ca^{2+} stores. *Proc Natl Acad Sci (USA)* **90**, 6259-6299.