Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release in Aplysia bag cell neurons requires interaction between mitochondrial and endoplasmic reticulum stores

by:

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Abstract

Intracellular Ca$^{2+}$ is influenced by both Ca$^{2+}$ influx and release. We examined intracellular Ca$^{2+}$ following action potential firing in the bag cell neurons of *Aplysia californica*. Following brief synaptic input, these neuroendocrine cells undergo an afterdischarge, resulting in elevated Ca$^{2+}$ and the secretion of neuropeptides to initiate reproduction. Cultured bag cell neurons were injected with the Ca$^{2+}$ indicator, fura-PE3, and subjected to simultaneous imaging and electrophysiology. Delivery of a 5 Hz, 1 min train of action potentials (mimicking the fast phase of the afterdischarge) produced a Ca$^{2+}$ rise that markedly outlasted the initial influx, consistent with Ca$^{2+}$-induced Ca$^{2+}$ release (CICR). This response was attenuated by about half with ryanodine or depletion of the endoplasmic reticulum (ER) by cyclopiazonic acid. However, depletion of the mitochondria, with carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, essentially eliminated CICR. Dual depletion of the ER and mitochondria did not reduce CICR further than depletion of the mitochondria alone. Moreover, tetraphenylphosphonium, a blocker of mitochondrial Ca$^{2+}$ release, largely prevented CICR. The Ca$^{2+}$ elevation during and subsequent to a stimulus mimicking the full afterdischarge was prominent and enhanced by PKC activation. Traditionally, the ER is seen as the primary Ca$^{2+}$ source for CICR. However, bag cell neuron CICR represents a departure from this view, in that it relies on store interaction, where Ca$^{2+}$ released from the mitochondria may in turn liberate Ca$^{2+}$ from the ER. This unique form of CICR may be used by both bag cell neurons, and other neurons, to initiate secretion, activate channels, or induce gene expression.
Introduction

Ca\(^{2+}\) channels are unique in that they conduct an electrical charge which is often translated into a biochemical signal (Hille 2003; Friel and Chiel 2008). Changes in Ca\(^{2+}\) can be amplified or prolonged through Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from intracellular stores (Endo et al. 1970; Fabiato and Fabiato 1975). Neuronal CICR was first seen in Aplysia (Gorman and Thomas 1980), then confirmed in bullfrog (Smith et al. 1983; Lipscombe et al. 1988) and rat (Neering and McBurney 1984). CICR is typically considered the product of voltage-gated Ca\(^{2+}\) influx opening ryanodine receptors (RyRs) on the endoplasmic reticulum (ER) to cause Ca\(^{2+}\) release (Bardo et al. 2005; Verkhratsky 2005). CICR is sensitive to the alkyloid, ryanodine (Meissner 1985; Imagawa et al. 1987), as well as depletion of ER Ca\(^{2+}\) by the Ca\(^{2+}\)-ATPase blocker, cyclopiazonic acid (CPA; Seidler et al. 1989), or the RyR agonist, caffeine (Weber 1968; Rousseau et al. 1988).

Upon entry from the extracellular space or the ER, mitochondria may take Ca\(^{2+}\) up via the mitochondrial Ca\(^{2+}\) uniporter (Gunter and Pfeiffer 1990; Gunter and Gunter 1994; Colegrove et al. 2000a,b; Kirichok et al. 2004; Kim et al. 2005). After sequestering Ca\(^{2+}\), mitochondria can release it back into the cytosol, typically through Na\(^+\)/Ca\(^{2+}\) and H\(^+\)/Ca\(^{2+}\) exchangers (Puskin et al. 1976; Wingrove and Gunter 1986; Gunter and Pfeiffer 1990; Pauceka and Jabůrek 2004; Colegrove et al. 2000a,b). Mitochondrial Ca\(^{2+}\) buffering and release is involved in post-tetanic potentiation (Tang and Zucker 1997; Zhong et al. 2001) and exocytosis (Giovannucci et al. 1999; Billups and Forsythe 2002).

Moreover, while not traditionally considered as a source for CICR, mitochondria have been found to modulate CICR (Friel and Tsien 1994; Jackson and Thayer 2006). In the present study, we further this role considerably by suggesting that mitochondrial Ca\(^{2+}\) release is a pre-requisite for CICR in the bag cell neurons of Aplysia.

The bag cell neurons from the marine mollusc, Aplysia californica, are neuroendocrine cells that control reproduction (Kupfermann 1967; Kupfermann and Kandel 1970; Pinsker and Dudek 1977;
Dudek et al. 1979). Following brief synaptic input, these neurons undergo a 30-min afterdischarge that triggers neuropeptide release and initiates egg-laying behaviour (Arch 1972a,b; Stuart et al. 1980; Loechner et al. 1990). The bag cell neurons have been employed to examine ion channels, peptide release, and intracellular Ca\(^{2+}\). For example, Fisher et al. (1994) used Ca\(^{2+}\)-sensitive electrodes to show that Ba\(^{2+}\) entry caused Ca\(^{2+}\) release, suggestive of CICR. In addition, both Ca\(^{2+}\) entry and release in these neurons have been linked to the synthesis and secretion of neuropeptides (Berry and Arch 1981; Loechner et al. 1990; Michel and Wayne 2002). Here, we demonstrate that bag cell neurons do in fact exhibit CICR, and that the underlying mechanism involves store interaction, i.e., following Ca\(^{2+}\) influx, mitochondrial Ca\(^{2+}\) release stimulates the ER to release Ca\(^{2+}\). More comprehensively, neurons may use store interaction to translate episodes of short-term electrical activity into long-term changes in intracellular Ca\(^{2+}\), thereby activating membrane conductances as well as trigger secretion, transcription, or translation.
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 approximately 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 MgCl₂ (around 50% of body weight), the abdominal ganglion was removed and treated for 18 h with neutral protease (13.33 mg/ml; 165859; Roche Diagnostics, Indianapolis, IN, USA) dissolved in tissue culture artificial seawater (tcASW; containing in mM: 460 NaCl, 10.4 KCl, 11 CaCl₂, 55 MgCl₂, 10 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 22°C 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, borosilicate 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.

**Ca\(^{2+}\) imaging**

The Ca\(^{2+}\)-sensitive dye, fura-PE3 (K\(^+\) salt; 0110; Teflabs, Austin, TX, USA; Vorndran *et al.* 1995), was pressure injected via sharp-electrode using a PMI-100 pressure microinjector (Dagan, Minneapolis, MN, USA), while simultaneously monitoring membrane potential with the Axoclamp. Microelectrodes (as per sharp-electrode current-clamp) had a resistance of 15-30 M\(\Omega\) when the tip was filled with 10 mM fura-PE3 then backfilled with 3 M KCl. Injections required 3-10, 0.2 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 subsequently for imaging showed resting potentials of -50 to -60 mV and displayed action potentials that overshot 0 mV following depolarizing current injection (0.5-1 nA, directly from the amplifier). After dye injection, neurons were allowed to equilibrate for at least 30 min. Imaging was performed using a Nikon TS100-F inverted microscope (Nikon, Mississauga, ON, Canada) equipped with Nikon Plan Fluor 20X (numerical aperture (NA) =0.5) or 40X (NA=0.6) objectives. The light source was a 75 W Xenon arc lamp and a multi-wavelength DeltaRAM V monochromatic illuminator (Photon Technology International, London, ON, Canada) coupled to the microscope with a UV-grade liquid-light guide. Excitation wavelengths were 340 and 380 nm. Between acquisition episodes, the excitation illumination was blocked by a shutter, which along with the excitation wavelength, was controlled by an IBM-compatible personal computer, a Photon Technology International computer interface, and ImageMaster Pro software (version 1.49, Photon Technology International). The emitted light passed through a 510/40 nm barrier filter prior to being detected by a Photon Technology International IC200 intensified charge coupled device camera. The camera intensifier voltage was set based on the initial fluorescence intensity of the cells at the beginning of each experiment and
maintained constant thereafter. The camera black level was set prior to an experiment using the camera controller such that, at a gain of 1, 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 sec 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\textsuperscript{2+}, and saved for subsequent analysis. The black level determination, image acquisition, frame averaging, emitted light ROI sampling, and ratio calculations were carried out using the ImageMaster Pro software.

**Reagents and drug application**

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 resting intracellular Ca\textsuperscript{2+} or Ca\textsuperscript{2+} transients evoked by action potential trains. The lack of an effect of DMSO is consistent with prior work by ourselves (Kachoei et al. 2006) and others (Jonas et al. 1997; Knox et al. 1992, 1996, 2004) who employ the bag cell neurons to study Ca\textsuperscript{2+} or Ca\textsuperscript{2+} channels. Tetraphenylphosphonium chloride (TTP; 218790; Sigma-Aldrich) was made up in water. Drug application or solution exchanges were accomplished by manual perfusion using a calibrated 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 (approximately 100 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. Pre-treatment duration was 20-30 min, unless stated otherwise.
Analysis

Origin (version 7; OriginLab Corporation, Northampton, MA, USA) was used to import and plot ImageMaster Pro files as line graphs. For intracellular Ca\textsuperscript{2+}, analysis usually compared the steady-state value of the baseline 340/380 ratio with the ratio from regions that had reached a peak or new steady-state during either action potential firing or CICR. For CICR, these regions are indicted in the figures by short dotted lines. Averages of both regions were determined by eye or with adjacent-averaging. Change was expressed as a percent change (peak % $\Delta$ 340/380 or CICR % $\Delta$ 340/380) of the new ratio over the baseline ratio. In one instance (Fig. 7C) the absolute change in the 340/380 during action potential firing was calculated. The time course of recovery for the Ca\textsuperscript{2+} signal was quantified by determining the time required, after delivery of the stimulus, for the 340/380 ratio to return to 75% of the baseline ratio observed before the stimulus. Summary data are presented as the mean ± standard error of the mean (SEM). Statistics were performed 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. Data were considered significantly different if the two-tailed p-value was <0.05.
Results

A prolonged train of action potentials is required for Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release

Previous studies of action potential-induced Ca\textsuperscript{2+}-influx in cultured bag cell neurons showed that both single spikes and bursts elevated intracellular Ca\textsuperscript{2+} (Fink et al. 1988; Knox et al. 1992; Fisher et al. 1994). However, in none of these cases were the rates and duration of the stimulus controlled to reflect physiological firing rates. Typically, the afterdischarge in the intact cluster is evoked by a 4-6 Hz, 10 sec train, which then results in two phases of action potential firing: a fast phase of approximately 1 min at 2-6 Hz, followed by a slow phase of close to 30 min at 0.5-1 Hz (Kaczmarek et al. 1982; Fisher et al. 1994; Magoski and Kaczmarek 2005; Zhang et al. 2002). Thus, for the present study, changes to intracellular Ca\textsuperscript{2+} were evoked with either a short train of action potentials corresponding to the stimulus used to trigger the afterdischarge, or a long train corresponding to the fast phase of the afterdischarge itself. In either case, we sought to determine if the Ca\textsuperscript{2+} rise produced by the stimuli resulted in subsequent intracellular Ca\textsuperscript{2+} release.

The short train of action potentials was delivered at 5 Hz for 10 sec to individual cultured bag cell neurons current-clamped to -60 mV. This elicited a distinct, fast-rising episode of Ca\textsuperscript{2+} influx, followed by a slow, essentially monoexponential decay back to baseline over the course of a few min (Fig. 1A; n=14). An endoplasmic reticulum Ca\textsuperscript{2+}-ATPase blocker, cyclopiazonic acid (CPA), was added before delivering the train to determine if the Ca\textsuperscript{2+} elevation was influenced by ER Ca\textsuperscript{2+} uptake and/or release. Following the 5 Hz, 10 s train, the peak percent change in Ca\textsuperscript{2+} for neurons treated with 20 \textmu M CPA was not statistically different from control (Fig. 1B,C; n=10). Similarly, the time to return to 75% of baseline Ca\textsuperscript{2+}, following administration of the train, was not significantly affected by CPA (Fig. 1D).

Because the Ca\textsuperscript{2+} change following the short train decayed quickly, and was not altered by depletion of the ER, a long train was employed. In this case, the stimulus consisted 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 this stimulus, followed by a slower, prolonged Ca$$^{2+}$$ elevation (Fig. 2A; n=6). This subsequent increase lasted several min and showed a distinct plateau suggestive of CICR. A hallmark of CICR is that Ca$$^{2+}$$ influx subsequently opens RyRs on the ER, thereby causing a secondary Ca$$^{2+}$$ rise. A potential role for the RyR-ER pathway in releasing and sustaining Ca$$^{2+}$$ was investigated by blocking RyRs prior to delivering the 5 Hz, 1 min train. Pre-treatment with 100 µM ryanodine significantly attenuated the amplitude of CICR by almost two-thirds when compared to control (Fig. 2B,C; n=5) without affecting the time to 75% recovery (data not shown).

**Depletion of the ER Ca$$^{2+}$$ store attenuates 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 to untreated neurons evoked characteristic rapid influx and subsequent CICR (Fig. 3A; 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. 3B,C; n=7). The time course of recovery to 75% of basal Ca$$^{2+}$$ was not altered (data not shown).

Although caffeine, an agonist of RyRs, has been used in other preparations to deplete the ER of Ca$$^{2+}$$ (Neering and McBurney 1984; Lipscombe et al. 1988; Orkand and Thomas 1995; Schmigol et al. 1995; Usachev and 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). Moreover, many bag cell neuron conductances are sensitive to adenosine 3’:5’-cyclic monophosphate (cAMP; Kaczmarek and Strumwasser 1984), and the inhibitory actions of caffeine on phosphodiesterases (Beavo et al. 1970) would confound matters by elevating cAMP levels.
Collapse of the mitochondrial membrane potential largely eliminates CICR

Although not considered requisite for CICR, other reports have suggested that mitochondrial buffering or release of Ca$^{2+}$ can influence CICR (Friel and Tsien, 1994; Werth and Thayer 1994; Jackson and Thayer 2006). Thus, the role of mitochondrial stores in bag cell neuron CICR was examined by applying carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) prior to delivering the stimulus. 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 and Prichard 1962; Simpson and Russell 1996; Collins et al. 2000). As expected, pre-treatment with FCCP before the stimulus resulted in robust Ca$^{2+}$ elevation. Moreover, in preliminary experiments, FCCP was seen to evoke a prominent depolarization coincident with depletion of mitochondrial Ca$^{2+}$. We have since learned that this depolarization appears to be caused by Ca$^{2+}$-dependent activation of a non-selective cation current (Hickey, Geiger and Magoski, in preparation). 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.

While delivery of a 5 Hz, 1 min train from -60 mV resulted in a robust Ca$^{2+}$ influx and CICR in nASW (Fig. 4A; n=7), pre-treatment with 20 µM FCCP significantly reduced CICR amplitude to one-fifth of the control amplitude (Fig. 4B,E; n=12). The time required to reach 75% recovery to baseline Ca$^{2+}$ was significantly shortened by FCCP (Fig. 4D). Provided CPA and FCCP act on separate Ca$^{2+}$ stores (see next section of Results), both of which appear to be involved in CICR, the response to the long train was examined following dual depletion. As was done before, neurons were pre-treated with 20 µM CPA to deplete the ER store. Once the fluorescence ratio intensity returned to baseline (20-40 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, with the result being a clear and significant reduction in
CICR (Fig. 4C; 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. 4E).

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

Key to suggesting that the effects of CPA and FCCP are disparate, is the certainty that they target separate Ca\(^{2+}\) stores. To confirm the ability of CPA to effectively deplete the ER, 20 µM 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 rise in Ca\(^{2+}\) followed by a return to near baseline. With subsequent addition of CaCl\(_2\) to the bath, a second prominent rise in Ca\(^{2+}\) was observed (Fig. 5A; n=11). This second Ca\(^{2+}\) elevation is referred to as store-operated Ca\(^{2+}\) influx, a process observed in a diversity of cell types and species. Store-operated Ca\(^{2+}\) influx only occurs when the ER has been depleted of Ca\(^{2+}\), resulting in a signal to the plasma membrane that opens Ca\(^{2+}\)-selective channels to replete the store (Putney 2003). Our laboratory has previously confirmed that bag cell neuron store-operated Ca\(^{2+}\) influx is pharmacologically and functionally the same as other systems (Kachoei et al. 2006). To determine whether the effects of CPA and FCCP depletion could be distinguished from one another, both agents were added in succession in Ca\(^{2+}\)-free ASW. After depleting the ER stores with 20 µM CPA and allowing recovery to near baseline Ca\(^{2+}\), 20 µM FCCP was added to the bath. The introduction of FCCP resulted in a second elevation of Ca\(^{2+}\) (Fig. 5B; n=13), thus signifying that the ER and the mitochondria are distinct Ca\(^{2+}\) stores.

**A blocker of mitochondrial Ca\(^{2+}\) release markedly reduces CICR**

FCCP decreases ATP synthesis as a result of dissipating the mitochondrial membrane potential (Abas et al. 2000; Allemann and Schneider 2000). Moreover, lowered ATP levels can reduce CICR (Abas et al. 2000; Murayama et al. 2000), presumably because of a loss of the direct stimulatory effect of ATP on the RyR channel (Rousseau et al. 1986). To rule out the possibility that a reduction in ATP was involved in decreasing CICR amplitude, the mitochondrial Ca\(^{2+}\) release blocker, tetraphenylphosphonium (TPP), was used. Unlike FCCP, TPP hinders mitochondrial Ca\(^{2+}\) release
without affecting ATP production (Aiuchi \textit{et al.} 1985). Specifically, TPP inhibits both the Na\textsuperscript{+}/Ca\textsuperscript{2+} and H\textsuperscript{+}/Ca\textsuperscript{2+} exchanger on the mitochondrial membrane, and thus blocks Ca\textsuperscript{2+} release without depleting the mitochondria of Ca\textsuperscript{2+} (Wingrove and Gunter 1986; Karodjov \textit{et al.} 1986).

In control cells, delivery of the 5 Hz, 1 min train triggered substantial Ca\textsuperscript{2+} influx and CICR (Fig. 6A; n=13). Consistent with other reports (Tang and Zucker 1997), pretreatment with 100 µM TPP did not change the membrane potential or intracellular Ca\textsuperscript{2+}. However, in the presence of TPP there was a significant reduction of CICR amplitude by nearly three-quarters, with only negligible effects on peak Ca\textsuperscript{2+} influx (Fig. 6B; n=5). TPP did not significantly change the duration of CICR (Fig 6D), despite a FCCP-like 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. 6C; n=11). That said, the decrease resulting from combined CPA/TPP treatment was not significantly different from TPP alone (Fig 6E). Thus, as was the case for FCCP, elimination of the mitochondrial store effectively eliminated CICR. We also attempted to use KBR 7943, a different Ca\textsuperscript{2+} exchanger antagonist (Iwamoto \textit{et al.} 1996); however, this drug blocked bag cell neuron Ca\textsuperscript{2+} current and prevented the initial influx.

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

Approximately 5 min following the onset of the afterdischarge in intact bag cell neurons, protein kinase C (PKC) activity is upregulated (Wayne \textit{et al.} 1999), which in turn enhances the voltage-gated Ca\textsuperscript{2+} current (DeReimer \textit{et al.} 1985; Strong \textit{et al.} 1987; Conn \textit{et al.} 1989a,b). Consequently, we tested the effects of phorbol 12-myristate 13-acetate (PMA), an effective PKC activator in bag cell neurons (Castagna \textit{et al.} 1982; Sossin and Schwartz 1994), on CICR. Delivery of the 5 Hz, 1 min train of action potentials in untreated neurons induced robust Ca\textsuperscript{2+} influx and CICR (Fig. 7A; n=7). In contrast, neurons pre-treated with 100 nM PMA for 25 min prior to the stimulus showed an approximately one-quarter reduction in peak Ca\textsuperscript{2+} influx during the train and no change in CICR amplitude (Fig. 7B-D; n=10). The Ca\textsuperscript{2+} influx during the train could have been lowered because of Ca\textsuperscript{2+}-dependent
inactivation of the Ca\(^{2+}\) current (Tillotson 1979). This form of use-dependent inactivation may have been increased due to a combination of the rapid stimulation and PKC-mediated enhancement of the Ca\(^{2+}\) current itself.

**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. However, *in vivo* the fast phase is followed by a prolonged period of slow firing (around 1 Hz for 30 min). Moreover, in the prior experiment involving PKC activation, PMA was added well before the train was delivered. Yet, in the intact cluster, PKC activity is not upregulated until approximately 5 min subsequent to the onset of the afterdischarge (Wayne *et al*. 1999). Thus, in an effort to recapitulate an afterdischarge-like stimulus, a compound train consisting of a 5 Hz, 1 min train (to mimic the fast phase), immediately followed by a 1 Hz, 30 min train (to mimic the slow phase) was delivered. Although the *in vivo* afterdischarge has a depolarizing component, we confined the stimulus to mimicking just action potential firing, so to limit both the extent of Ca\(^{2+}\) current inactivation (which would speed up if evoked repeatedly from a more depolarized potential) and the activation of any persistent voltage-dependent Ca\(^{2+}\) or cation channels (which would provide additional, uncontrolled Ca\(^{2+}\) influx). In addition, PMA was applied to some neurons just 10 min prior to the compound train, such that the kinetics for PKC activation would be more physiological. Conn *et al*. (1989b) showed that bath-applied PMA increased Ca\(^{2+}\) current in cultured bag cell neurons within 10-15 min.

Delivery of the simulated afterdischarge produced a biphasic Ca\(^{2+}\) elevation. This included an initial spike of Ca\(^{2+}\) during the 5Hz, 1 min phase, followed by a slowly-declining plateau through the course of the 1 Hz, 30 min phase (Fig. 8A; n=10). The Ca\(^{2+}\) elevation was quantified by taking the percent change from baseline over the early and late periods of the 1 Hz, 30 min phase. Upon termination of the compound train, the Ca\(^{2+}\) returned to baseline relatively quickly, with a time to 75% recovery of close to 5 min (Fig. 8E). Remarkably, this appeared to be similar to, or even faster than, that
seen following CICR induced by the 5Hz, 1 min train alone (compare to Fig. 4D or 6D). Treatment with 100 nM PMA 10 min prior to the compound train significantly enhanced the Ca\(^{2+}\) elevation (Fig. 8B; n=7). In comparison to control, the Ca\(^{2+}\) change during both the early and late period of the 30 min, 1 Hz phase was nearly a third-again as large with PMA. Moreover, PKC activation significantly prolonged the return to baseline duration, with almost a four-fold increase in the time to 75% recovery (Fig. 8E).
Discussion

A short train of action potentials, like the stimulus typically used to trigger an afterdischarge in vivo (5 Hz, 10 s), failed to initiate CICR in cultured bag cell neurons. Rather, CICR required a burst of action potentials mimicking the fast phase of the afterdischarge itself (5 Hz, 1 min). This is similar to certain forms of neuronal CICR evoked by only lengthy bursts of action potentials and prominent Ca\(^{2+}\) influx (Smith et al. 1983; Neering and McBurney 1984; Schmigol et al. 1995; Richter et al. 2005), but is unlike other versions where brief firing or single action potentials are sufficient to initiate CICR (Gorman and Thomas 1980; Hua et al. 1993; Orkand and Thomas 1995; Cohen et al. 1997; Solovyova et al. 2002). Because the response in bag cell neurons occurs only subsequent to the fast phase-like train, it would suggest that Ca\(^{2+}\) release is more likely once the afterdischarge itself is underway, rather than just after the initial stimulation. This may serve to avoid erroneous Ca\(^{2+}\) release.

In part, bag cell neuron CICR is consistent with similar phenomena observed in other preparations. First, it is reduced by ryanodine, which acts on RyRs found on the ER (Berridge 1998; Meldolesi 2001; Bardo et al. 2006). Typically between 50 and 100 µM is sufficient to eliminate CICR, as reported in leech (Trueta et al. 2004), salamander (Suryanarayanan and Slaughter 2006), rat dorsal root ganglion (DRG; Shmigol et al. 1995), frog sympathetic (Hua et al. 1993), and Hermissenda (Kawai et al. 2004) neurons. The present result is in agreement with RyRs playing a role in generating bag cell neuron CICR. Second, depletion of CPA-sensitive stores also reduces CICR in a manner much like that of ryanodine. This is further indicative of a RyR-ER pathway, and is similar to that observed in Helix (Orkand and Thomas 1995), rat DRG (Usachev and Thayer 1997), rat hippocampal (Emptage et al. 1999), and rat thalamic (Richter et al. 2005) neurons. Nevertheless, given that bag cell neuron CICR is only partially blocked by either a high concentration of ryanodine or ER depletion, it suggests a secondary mechanism may be at work. It is unlikely that either ryanodine or CPA impact other Ca\(^{2+}\) pumps or exchangers in a non-specific manner. For example, ryanodine does not alter basal cytoplasmic
Ca\(^{2+}\) in our preparation or others (Hua et al. 1993; Ivanenko et al. 1993; Kang and Holz 2003), and CPA does not block the plasma membrane Ca\(^{2+}\)-ATPase (Seidler et al. 1989). Similarly, we are confident that the bag cell neuron ER is actually depleted by CPA because not only does it result in store-operated Ca\(^{2+}\) influx, but it also eliminates IP\(_3\)-induced Ca\(^{2+}\) elevations (Jonas et al. 1997) and liberates the Ca\(^{2+}\) that remains in the ER after RyR activation by other agonists (Kachoei et al. 2006).

Bag cell neuron CICR is unique in that it is nearly eliminated by depletion of the mitochondrial store with FCCP, or preventing mitochondrial Ca\(^{2+}\) release with TPP. Moreover, simultaneous ER depletion by CPA does not reduce CICR further. Thus, although supported by Ca\(^{2+}\) from the ER, CICR appears to depend on mitochondrial Ca\(^{2+}\). FCCP both depletes mitochondria of Ca\(^{2+}\) and lowers oxidative phosphorylation by collapsing the mitochondrial membrane potential (Heytler and Prichard 1962; Collins et al. 2000). Because a decrease in ATP levels can inhibit the RyR channel (Rousseau et al. 1986), FCCP could suppress CICR indirectly. To eliminate this uncertainty, TPP, which blocks Ca\(^{2+}\) release from mitochondria (Wingrove and Gunter 1986) with little effect on ATP production (Aiuchi et al. 1985), was employed. TPP also appears to be specific, as it does not change basal Ca\(^{2+}\) or membrane potential in our preparation or the presynaptic terminals of crayfish motor neurons (Tang and Zucker 1997) and rat Caylx of Held (Billups and Forsythe 2002), which might be the case if it blocked Ca\(^{2+}\) pumps or exchangers not on the mitochondria. For bag cell neurons, the FCCP and TPP results are essentially identical, and suggest that mitochondrial Ca\(^{2+}\) release is required for CICR.

There is evidence from other preparations that mitochondria can influence CICR. For example, when Werth and Thayer (1994) uncoupled mitochondria and prevented electron transport, it blocked depolarization-induced Ca\(^{2+}\) release in rat DRG neurons. They suggest that mitochondria exclusively 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 CICR-like fashion. This sustained Ca\(^{2+}\) release was prevented by FCCP but not altered by ryanodine or ER depletion with
thapsigargin (Jackson et al. 1988; Colegrove et al. 2000a). Friel and Tsien (1992) also observed true CICR in these neurons, which was blocked by depletion with caffeine. Essentially, these studies concluded that a large Ca\(^{2+}\) influx engaged mitochondria, while a modest Ca\(^{2+}\) influx engaged ER, with no interaction between the two.

However, bag cell neuron CICR hinges on the mitochondria, which raises the possibility of an interaction between mitochondrial and ER stores, as proposed by Jackson and Thayer (2006). They showed that both ATP and Ca\(^{2+}\) released from mitochondria influence the frequency and magnitude of caffeine-induced, RyR-dependent Ca\(^{2+}\) oscillations in rat DRG neurons. But is this the case for CICR in bag cell neurons? There are two plausible explanations for the apparent dependence of bag cell neuron CICR on mitochondria. First, 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 RyRs, which in turn causes Ca\(^{2+}\) release from the ER (CPA and ryanodine sensitive). Depletion of the ER (with CPA) or blocking RyRs (with ryanodine) does not eliminate CICR, because mitochondrial release would also contribute to the plateau. However, depletion of mitochondrial Ca\(^{2+}\) (with FCCP) or elimination of its release (with TPP) impedes both mechanisms. Second, 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.

FCCP may have changed bag cell neuron ATP levels, which could in turn affect RyRs or other ATP- or proton-dependent mechanisms, but this is not supported by the TPP result. Furthermore, FCCP-treated neurons recover from the 5 Hz, 1 min train-induced Ca\(^{2+}\) influx in a manner similar to controls, suggesting a non-specific effect on Ca\(^{2+}\) handling is unlikely. 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. CICR is initiated and supported by Ca\(^{2+}\) buffering and release from the mitochondria, with a subsequent contribution from the ER, but without ATP-RyR interactions. This represents a
departure from the classic model of CICR where Ca\(^{2+}\) influx activates RyRs directly and the mitochondria merely buffer Ca\(^{2+}\) entry (Fig. 9).

Upon initial stimulation, and during the fast phase of the afterdischarge, bag cell neuron Ca\(^{2+}\) influx occurs through a basal Ca\(^{2+}\) channel (DeRiemer et al. 1985). However, during the slow phase of the afterdischarge, PKC is activated and a larger-conductance, PKC-sensitive channel is inserted into the plasma membrane (DeRiemer et al. 1985; Conn et al. 1987; Strong et al. 1987; Wayne et al. 1999; White and Kaczmarek 1997; Zhang et al. 2008). Attempts at enhancing Ca\(^{2+}\) influx during the fast phase alone with PMA pre-treatment were unsuccessful. This may have been due to a combination of greater initial Ca\(^{2+}\) and the high firing rate causing more Ca\(^{2+}\)-dependent inactivation of the Ca\(^{2+}\) current, and less overall Ca\(^{2+}\) influx. In many respects, this protocol fails to replicate natural phenomena; thus, PMA application was timed to elevate PKC activity several min into the slow phase of a compound train that mimicked the firing frequency of the afterdischarge. PKC activation resulted in a prominent increase in the overall amount of Ca\(^{2+}\) during the slow phase. Moreover, post-train recovery time was clearly lengthened by PMA as compared to control. This suggests that CICR may have occurred subsequent to the compound train. Other factors contributing to this prolonged post-compound train signal include the larger Ca\(^{2+}\) load from PKC-dependent upregulation of the Ca\(^{2+}\) current during the slow phase and possible effects of PKC on Ca\(^{2+}\) removal (Usachev et al. 2006).

That bag cell neurons have voltage-gated Ca\(^{2+}\) influx renders CICR somewhat enigmatic. Possibly, CICR is needed to amplify or intensify peptide secretion. For example, CICR augments acetylcholine release from Aplysia buccal neurons (Mothet et al. 1998, Chameau et al. 2001), serotonin from leech Retzius neurons (Trueta et al. 2004), glutamate from salamander photoreceptors (Suryanarayanan and Slaughter 2006), and insulin from a beta cell line (Kang and Holz 2003). Moreover, Michel and Wayne (2002) found that bag cell neuron peptide secretion could persist well beyond the end of the afterdischarge. Perhaps, this is due to the very prolonged recovery of intracellular Ca\(^{2+}\) we observed after the compound train in PMA-treated neurons. Postulations aside, bag cell neuron
CICR should be considered in the context that our recordings were made from single neurons in culture at room temperature. *In vivo*, these cells are electrically coupled to one another and typically operate at several degrees below room temperature. Thus, the kinetics of Ca\(^{2+}\) influx, release, and handling may not be identical in the intact animal.

Bag cell neuron CICR may also activate membrane conductances contributing to the depolarizing drive for the afterdischarge, such as Ca\(^{2+}\)-dependent, non-selective cation channels (Magoski *et al.* 2002; Lupinsky and Magoski 2006; Hung and Magoski 2007). This would be important during the slow phase, when voltage-gated Ca\(^{2+}\) influx is lessened. Our laboratory has observed cation channel activation subsequent to mitochondrial Ca\(^{2+}\) release (Hickey, Geiger and Magoski, in preparation), the latter of which appears to be critical for CICR. In chick DRG neurons, CICR activates Cl\(^{-}\) channels (Ivanenko *et al.* 1993), while in rat hippocampal neurons, RyR blockade attenuates cation channel-mediated depolarization (Partridge and Valenzuela 1999).

Finally, CICR may impact several forms of Ca\(^{2+}\)-dependent plasticity occurring during the afterdischarge. Such changes include the induction of the refractory period (Kaczmarek *et al.* 1982; Kaczmarek and Kauer 1983; Magoski *et al.* 2000) and enhanced translation of peptide hormone (Berry and Arch 1981; Wayne *et al.* 2004). These events may be mediated by CaM-kinase, which has increased activity during the afterdischarge (DeRiemer *et al.* 1984). CICR influences plasticity in other systems; for example, conditioning-mediated changes to *Hermissenda* photoreceptor excitability and morphology are RyR-dependent (Blackwell and Alkon 1999; Kawai *et al.* 2004). In hippocampal dendrites, CICR triggered by Ca\(^{2+}\) influx through NMDA receptors, has a role in inducing some forms of long-term potentiation and depression (Harvey and Collingridge 1992; Reyes and Stanton 1996; Emptage *et al.* 1999). Thus, there is the potential for mitochondrial/ER-dependent CICR in bag cell neurons to augment neurosecretion, enhance the depolarization necessary for secretion, or trigger plasticity.
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Figure legends

Figure 1. A short train of action potentials fails to evoke CICR in cultured bag cell neurons

A) Simultaneous recordings of 340/380 fura PE3 fluorescence signal and membrane potential in nASW (11 mM Ca²⁺). The upper trace shows Ca²⁺ influx indicated by the change in the intensity of the 340/380 ratio following a 5 Hz, 10 s train. Baseline 340/380 is 0.357. Fluorescence ratio scale bar applies to both A and B. The lower traces depicts membrane potential. A typical recording of action potentials appears as a thick vertical bar evoked by the train.

B) A somewhat smaller response 25 min after pretreatment with 20 µM CPA, an inhibitor of SERCA. Baseline 340/380 is 0.349.

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

D) The time required to reach 75% recovery of baseline Ca²⁺ appears extended by CPA; however, this difference is not significant (Mann-Whitney U test).

Figure 2. 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 Ca²⁺ elevation, followed by a slow plateau that greatly outlasts the stimulus. This second phase of Ca²⁺ elevation displays characteristics consistent with CICR (at arrow). Baseline 340/380 is 0.392. The fluorescence ratio scale applies to panels A and B. In this and all subsequent Ca²⁺ traces concerning CICR, a short dotted line indicates the region used to calculate the percent change in 340/380 as compared to the baseline ratio (see Materials and Methods).

B) Following treatment with 100 µM ryanodine, the second phase of the Ca²⁺ elevation is markedly reduced. Baseline 340/380 is 0.303.
C) Summary graph indicating that prior application of ryanodine significantly attenuates CICR amplitude (unpaired t test).

**Figure 3. Inhibition of SERCA attenuates CICR.**

A) Response to the 5 Hz, 1 min train of action potentials shows CICR. Baseline 340/380 is 0.431. The scale bar for the fluorescence ratio applies to both A and B.

B) Prior store depletion by 20 μM CPA reduces CICR evoked by the long train. Baseline 340/380 is 0.450.

C) Summary data showing depletion of the CPA sensitive store significantly reduces the amplitude of CICR (Mann-Whitney U test).

**Figure 4. Dual depletion by CPA and FCCP lacks additive inhibition of CICR.**

A) CICR evoked by a 5 Hz, 1 min train. Baseline 340/380 is 0.451. Ratio scale bar applies to panels A-C.

B) Addition of 20 μM FCCP prior to delivery of the long train clearly abates CICR. Baseline 340/380 is 0.584.

C) Consecutive depletion by 20 μM CPA and FCCP also reduces CICR amplitude. Baseline 340/380 is 0.570.

D) FCCP significantly reduces time to 75 % recovery (Mann-Whitney U test).

E) Summary data comparing CICR amplitude under control and following FCCP or CPA plus FCCP. The amplitude of CICR is significantly reduced in FCCP alone and when combined with CPA. However, CICR following dual depletion does not differ from FCCP alone (Kruskal-Wallis ANOVA and Dunn’s post-hoc test).
Figure 5. CPA and FCCP deplete separate Ca\(^{2+}\) stores.

A) Response to Ca\(^{2+}\) addition following 20 \(\mu\)M 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 near baseline. The addition of 11 mM extracellular Ca\(^{2+}\) (CaCl\(_2\)) results in an additional rise in Ca\(^{2+}\). 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). Baseline 340/380 is 0.374. Representative of \(n=11\).

B) Effect of 20 \(\mu\)M FCCP on Ca\(^{2+}\) following depletion of the ER stores with 20 \(\mu\)M CPA in Ca\(^{2+}\)-free ASW. Following the initial rise induced by CPA, the Ca\(^{2+}\) returns to near baseline, and the neuron is depleted a second time with FCCP. FCCP induces a second rise in Ca\(^{2+}\), indicating a separate FCCP-sensitive Ca\(^{2+}\) store. Baseline 340/380 is 0.315. Representative of \(n=13\).

Figure 6. TPP is as effective in reducing CICR as CPA plus TPP.

A) CICR evoked by a 5 Hz, 1 min train. Baseline 340/380 is 0.318. Ratio scale bar applies to panels A-C.

B) Pretreatment with 100 \(\mu\)M TPP noticeably decreases CICR. Baseline 340/380 is 0.341.

C) Following 20 \(\mu\)M CPA and TPP, CICR amplitude is also markedly reduced. Baseline 340/380 is 0.310.

D) TPP appears to decrease time to 75% recovery, but not significantly (Mann-Whitney U test).

E) Summary data comparing amplitude of CICR under control and following TPP or TPP plus CPA. The addition of TPP significantly reduces CICR, but there is no difference between TPP and TPP plus CPA (Kruskal-Wallis ANOVA and Dunn’s post-hoc test).
Figure 7. Peak Ca\textsuperscript{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. Baseline 340/380 is 0.366. The scale bar for fluorescence ratio applies to both panels A and B.

B) A 25 min pretreatment with 100 nM PMA does not alter CICR amplitude. However, PKC activation does decrease the amplitude of the initial Ca\textsuperscript{2+} influx. Baseline 340/380 is 0.473.

C) Peak Ca\textsuperscript{2+} influx is modestly but 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 8. Ca\textsuperscript{2+} is enhanced by a simulated afterdischarge delivered shortly after PKC activation

A) Representative of Ca\textsuperscript{2+} measured in response to a compound train of action potentials simulating the afterdischarge (5 Hz, 1 min continuous with 1 Hz, 30 min). Following the sharp increase during the 5 Hz 1 min phase, the Ca\textsuperscript{2+} pattern shows a large-amplitude component that declines over the course of the 1 Hz, 30 min phase. Periods used to determine the Ca\textsuperscript{2+} amplitude during the 1 Hz, 30 min phase are marked as early (E) and late (L). Baseline 340/380 is 0.357. Ratio scale bar applies to panels A and B.

B) Effect of 100 nM PMA addition (at arrow), 10 min prior to the delivery of the compound train. PMA results in a greater initial Ca\textsuperscript{2+} elevation, increased Ca\textsuperscript{2+} during both the early and late periods of the slow phase, less of a decline during the course of the slow phase, and a much longer time to recovery after the train. A short, spontaneous burst of action potentials occurs approximately 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 and Magoski unpublished). Baseline 340/380 is 0.341.

C and D) Summary data comparing the percent change in Ca\textsuperscript{2+} amplitude taken from the early and late periods of the slow phase. During both periods, PMA results in a significant increase in Ca\textsuperscript{2+} compared to control (unpaired t test).
E) Summary data for the time to reach 75% recovery to baseline. The addition of PMA significantly lengthens the time to reach 75% recovery (unpaired t test). In two controls, the impalement was lost before 75% recovery was reached; thus, the n-value decreases from 10 in panels C and D to 8 in panel E.

**Figure 9. Traditional vs. bag cell neuron CICR**

A) A schematic showing the traditional model for 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) and Ca$^{2+}$ is released from the endoplasmic reticulum (ER; step 3). Ca$^{2+}$ entry may be buffered by mitochondria (MIT) via the mitochondrial Ca$^{2+}$ uniporter (MCU; step 2a).

B) An updated schematic for CICR based on the bag cell neurons. Ca$^{2+}$ influx occurs via voltage-gated Ca$^{2+}$ channels (step 1), and upon entry is taken up by the mitochondria through the MCU (step 2). Ca$^{2+}$ is then released from the mitochondria though cation exchangers (CaXΔ; step 3). Subsequently, this released Ca$^{2+}$ stimulates RyRs on the ER membrane, producing CICR (step 4). In addition, Ca$^{2+}$ release from mitochondria directly contributes to CICR.
Figure 1

A. Control

B. 20 µM CPA

C. Peak % Δ340/380

D. Time to 75% recovery (min)
Figure 2

A  Control

B  100 μM ryanodine

C  CICR % Δ 340/380

p<0.03
Figure 3

A. Control

B. 20 µM CPA

C. CICR% Δ340/380

-60 mV

50 mV

1 min

2 min

0.2 (340/380)

40 mV

p<0.03

17

7

Ctrl

CPA
Figure 4

A. Control

B. 20 μM FCCP

C. 20 μM CPA + 20 μM FCCP

D. Time to 75% recovery (min)

E. CICR % (340/380)

- 60 mV

- 50 mV

- 40 mV

- 30 mV

- 20 mV

- 10 mV

- 0 mV

p<0.03

p<0.01

ns
Figure 5

A

in Ca$^{2+}$-free ASW

B

in Ca$^{2+}$-free ASW

$0.1$ (340/380)

10 min

20 $\mu$M CPA

20 $\mu$M FCCP
Figure 6

A  Control

B  100 µM TPP

C  20 µM CPA + 100 µM TPP

D  Time to 75% recovery (min)

E  CICR % Δ 340/380

Ctrl  TPP  CPA + TPP

13  5
15  5  11

p<0.01  p<0.05  ns
Figure 7

A. Control

B. 25 min pretreat 100 nM PMA

C. Peak Δ340/380

D. CICR % Δ340/380

- p<0.03

- ns
Figure 8

A

Control

Early slow phase %

D 340/380

PMA

Time to 75% recovery (min)

B

100 nM PMA

Late slow phase %

D 340/380

PMA

C

Early slow phase % Δ 340/380

Control 10 PMA 7

p<0.05

D

Late slow phase % Δ 340/380

Control 10 PMA 7

p<0.05

E

Time to 75% recovery (min)

Control 8 PMA 7

p<0.05
Figure 9

A

Traditional CICR

1

Ca$^{2+}$

VGCC

2

Ca$^{2+}$

MCU

2b

Ca$^{2+}$

RyR

3

Ca$^{2+}$

MIT

Ca$^{2+}$

ER

B

Bag cell neuron

CICR

1

Ca$^{2+}$

VGCC

2

Ca$^{2+}$

MCU

2b

Ca$^{2+}$

CaX$\Delta$

3

Ca$^{2+}$

RyR

4

Ca$^{2+}$

MIT

Ca$^{2+}$

ER