Mitochondrial Ca\(^{2+}\) Activates a Cation Current in *Aplysia* Bag Cell Neurons

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Mitochondrial Ca\(^{2+}\) activates a cation current in *Aplysia* bag cell neurons. J Neurophysiol 103: 1543–1556, 2010. First published January 13, 2010; doi:10.1152/jn.01121.2009. Ion channels may be gated by Ca\(^{2+}\) entering from the extracellular space or released from intracellular stores—typically the endoplasmic reticulum. The present study examines how Ca\(^{2+}\) impacts ion channels in the bag cell neurons of *Aplysia californica*. These neuroendocrine cells trigger ovulation through an afterdischarge involving Ca\(^{2+}\) influx from Ca\(^{2+}\) channels and Ca\(^{2+}\) release from both the mitochondria and endoplasmic reticulum. Mitochondrial Ca\(^{2+}\) with the protonophore, carbonyl cyanide-4-trifluoromethoxyphenyl-hydrazone (FCCP), depolarized bag cell neurons, whereas depleting endoplasmic reticulum Ca\(^{2+}\) with the Ca\(^{2+}\)-ATPase inhibitor, cyclopiazonic acid, did not. In a concentration-dependent manner, FCCP elicited an inward current associated with an increase in conductance and a linear current/voltage relationship that reversed near −40 mV. The reversal potential was unaffected by changing intracellular Cl\(^{−}\), but left-shifted when extracellular Ca\(^{2+}\) was removed and right-shifted when intracellular K\(^{+}\) was decreased. Strong buffering of intracellular Ca\(^{2+}\) decreased the current, although the response was not altered by blocking Ca\(^{2+}\)-dependent proteases. Furthermore, fura imaging demonstrated that FCCP elevated intracellular Ca\(^{2+}\) with a time course similar to the current itself. Inhibiting either the V-type H\(^{+}\)-ATPase or the ATP synthase failed to produce a current, ruling out acidic Ca\(^{2+}\) stores or disruption of ATP production as mechanisms for the FCCP response. Similarly, any involvement of reactive oxygen species potentially produced by mitochondrial depolarization was mitigated by the fact that dialysis of xanthine/xanthine oxidase did not evoke an inward current. However, both the FCCP-induced current and Ca\(^{2+}\) elevation were diminished by disabling the mitochondrial permeability transition pore with the alkylating agent, N-ethylmaleimide. The data suggest that mitochondrial Ca\(^{2+}\) gates a voltage-independent, nonselective cation current with the potential to drive the afterdischarge and contribute to reproduction. Employing Ca\(^{2+}\) from mitochondria, rather than the more common endoplasmic reticulum, represents a diversification of the mechanisms that influence neuronal activity.

**INTRODUCTION**

Intracellular Ca\(^{2+}\) is a fundamental signal that impacts secretion, gene expression, and ion channel function (Greer and Greenberg 2008; Levitan 1999; Neher and Sakaba 2008). Ca\(^{2+}\) enters neurons through voltage-gated Ca\(^{2+}\) channels as well as via release from intracellular stores (Friel and Chiel 2008). The best-studied store is the endoplasmic reticulum, which may be accessed by Ca\(^{2+}\) influx stimulating ryanodine receptors (Ca\(^{2+}\)-induced Ca\(^{2+}\) release), metabotropic receptors initiating inositol phosphate synthesis, or block of the Ca\(^{2+}\)-ATPase [by cyclopiazonic acid (CPA) or thapsigargin] permitting Ca\(^{2+}\) to leak out of the organelle (Bardo et al. 2006; Seidler et al. 1989; Solovyova et al. 2002; Thastrup et al. 1990). Endoplasmic reticulum Ca\(^{2+}\) can influence neuronal activity and excitability by acting on various ion channels, including Ca\(^{2+}\)-activated K\(^{+}\) and nonselective cation channels (Crawford et al. 1997; Li et al. 1999; Tatsumi and Katayama 1994) as well as ionotropic receptors and voltage-gated Ca\(^{2+}\) channels (Empetge et al. 2001; Richter et al. 2005).

The other major reservoir is the mitochondria, which maintains high Ca\(^{2+}\) because the very negative voltage across the inner membrane makes Ca\(^{2+}\) entry electrically favorable (Campanella et al. 2004). Ca\(^{2+}\) can be liberated by collapsing this voltage with protonophores such as carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (Fulceri et al. 1991; Heytler and Pribram 1962). Additionally, loading mitochondria with Ca\(^{2+}\) during action potentials can lead to subsequent release of that Ca\(^{2+}\) through exchangers (Colegrove et al. 2000; Wingrove and Gunter 1986). Mitochondria have been considered Ca\(^{2+}\) buffers or sinks with a less-than-direct role in Ca\(^{2+}\) signaling (Friel and Tsien 1986). Thus, while mitochondrial Ca\(^{2+}\) uptake certainly influences synaptic transmission and plasticity (Billups and Forsythe 2002; Tang and Zucker 1997), there is little evidence of mitochondrial Ca\(^{2+}\) itself accessing membrane channels and regulating excitability.

The bag cell neurons control reproduction in the marine mollusk, *Aplysia californica* (Kupfermann 1967; Pinsker and Dudek 1977). Stimulation of these neurons initiates a prolonged burst, referred to as the afterdischarge, which culminates in the neurohemal secretion of peptide hormones to trigger egg-laying behavior (Conn and Kaczmarek 1989). We have previously reported that the bag cell neurons present a unique form of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Geiger and Magoski 2008). This response is partially attenuated by depleting endoplasmic reticulum Ca\(^{2+}\) with CPA or blocked altogether by prior pharmacological treatments causing either mitochondrial depolarization (with FCCP) or inhibition of mitochondrial Ca\(^{2+}\) exchange. It appears that subsequent to voltage-gated Ca\(^{2+}\) entry, the mitochondria expel their Ca\(^{2+}\) to elicit further Ca\(^{2+}\) release from the endoplasmic reticulum. The present study examines the biophysical consequence of liberating Ca\(^{2+}\) from either the mitochondria (with FCCP) or the endoplasmic reticulum (with CPA) in bag cell neurons. Our rather surprising finding is that Ca\(^{2+}\) from the mitochondria, rather than the endoplasmic reticulum, causes marked depolarization by opening a cation channel. Not only do these results have implications for the afterdischarge and *Aplysia* reproduction, but broaden the general function of the mitochondria from a somewhat passive store to one capable of actively regulating membrane conductances.

**METHODS**

**Animals and cell culture**

Primary cultures of isolated bag cell neurons were attained from adult *A. californica*, weighing 150–500 g. Animals were purchased...
from Marinus (Long Beach, CA) or Santa Barbara Marine Biologicals (Santa Barbara, CA) and housed in an ~300 l aquarium containing continuously circulating, aerated artificial seawater (Instant Ocean; Aquarium Systems; Mentor, OH) at 14–16°C on 12/12-h light/dark cycle and fed Romaine lettuce 5 times per week. Following anesthesia by injection of isotonic MgCl₂ (~50% of body weight), the abdominal ganglion was removed and treated for 18 h with neutral protease (13.33 mg/ml; 165859; Roche Diagnostics, Indianapolis, IN) dissolved in tissue culture artificial seawater (tcASW; containing, in mM, 460 NaCl, 10.4 KCl, 1 CaCl₂, 55 MgCl₂, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mg/ml glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, pH 7.8 with NaOH). The ganglion was then transferred to fresh tcASW, and the two bag cell neuron clusters dissected from the surrounding connective tissue. Using a fire-polished Pasteur pipette and gentle trituration, neurons were dispersed in tcASW onto 35 × 10 mm polystyrene tissue culture dishes (430165; Corning, Corning, NY, or 353001; Falcon Becton-Dickinson, Franklin Lakes, NJ). Cultures were maintained in either tcASW or, on occasion, simple culture medium [SCM; composition as per tcASW plus minimum essential medium (MEM) vitamins (0.5×; 11120052; Gibco/Invitrogen; Grand Island, NY), MEM nonessential amino acids (0.2×; 11040050; Gibco/Invitrogen), and MEM essential amino acids without t-glutamine (0.2×; 1130051; Gibco/Invitrogen)] for 1–3 days in a 14°C incubator. Experiments were carried out at 22°C. Salts were obtained from Fisher Scientific (Ottawa, ON, Canada), ICN (Irvine, CA), or Sigma-Aldrich (St. Louis, MO).

**Whole cell, voltage-clamp recordings**

Voltage-clamp recordings were made using an EPC-8 amplifier (HEKA Electronics; Mahone Bay, NS, Canada) and the whole-cell method. Microelectrodes were pulled from 1.5 mm internal diameter, borosilicate glass capillaries (TW150F-4; World Precision Instruments, Sarasota, FL) and had a resistance of 1–3 MΩ when filled with intracellular saline. Pipette junction potentials were nulled immediately before seal formation. Pipette and neuronal capacitive current were canceled and the series resistance (3–5 MΩ) was compensated to 70–80% and monitored throughout the experiment. Cell capacitance was derived from the EPC-8 whole cell capacitance compensation. Current was filtered at 1 kHz by the EPC-8 Bessel filter and monitored with the Axoclamp. Microelectrodes (as per sharp electrode, current clamp) had a resistance of 15–30 MΩ when tip-filled with 10 mM fura-PE3 and backfilled with 3 M KCl. Currents recorded were filtered at 1 kHz and displayed on a digital oscilloscope with a storage function set to capture two consecutive traces. Biases were selected such that they were near the resting membrane potential, which was set to approximately the middle of the neuron. The ratio of fluorescence intensity was controlled by an IBM-compatible personal computer, a Digidata 1300 A/D converter (Axon Instruments/Molecular Devices; Sunnyvale, CA) and the Clampex acquisition program of pCLAMP 8.1 (Axon Instruments). Clampex was also used to control the membrane potential. Recordings were done in normal ASW (nASW; composition as per tcASW but with glucose and antibiotics omitted) or Ca²⁺-free ASW [composition as per tcASW but with added 0.5 mM ethylene glycol bis(aminohexyl) ether] tetracetic acid (EGTA) as well as omitting glucose, antibiotics, and Ca²⁺]. Usually, microelectrodes were filled with regular intracellular saline [composition in mM: 500 K-aspartate, 70 KCl, 1.25 MgCl₂, 10 HEPES, 10 glucose, 1 glutathione, 5 ATP (grade 2, disodium salt; Sigma), and 0.1 GTP (type 3, disodium salt; Sigma); pH 7.3 with KOH; free Ca²⁺ concentration almost always set to 300 nM, but in a few instances set to 1 µM]. In some experiments, intracellular Ca²⁺ was buffered using a high-EGTA intracellular saline with 20 mM EGTA, 5 mM MgCl₂, and a free Ca²⁺ concentration set at 35 nM. Ca²⁺ concentrations were calculated using WebMaxC (http://www.stanford.edu/~cpaton/webmaxC.html). Both intracellular salines had a calculated junction potential of 15 mV versus ASW, which was compensated by off-line subtraction. For ion substitution, a low K⁺ intracellular saline (composition as per regular saline but with the K-aspartate replaced with N-methyl-d-glucamine and aspartic acid; junction potential of 9 mV vs. nASW) and a high Cl⁻ internal (composition as per regular saline but with the K-aspartate replaced with KCl) were used.

**Current-clamp recording**

Current-clamp recordings were made using either the EPC-8 and the whole cell current-clamp method or an Axoclamp 2B amplifier (Axon Instruments/Molecular Devices) and the sharp-electrode, bridge-balanced method. Microelectrode and intracellular solution for whole cell, current-clamp were as per whole cell, voltage-clamp. Microelectrodes for sharp-electrode current-clamp were pulled from 1.2 mm internal diameter, borosilicate glass capillaries (IB120F-4; World Precision Instruments) and had a resistance of 7–12 MΩ when filled with 2 M K-acetate (supplemented with 100 mM KCl and 10 mM HEPES; pH = 7.3 with KOH). Voltage signals were filtered at 3 kHz and displayed at 2 kHz. Current was delivered either from the amplifier, Clampex, or a S88 stimulator (Grass; Warwick, MA).

**Ca²⁺ imaging**

The Ca²⁺-sensitive dye, fura-PE3 (K⁺ salt; 0110; Teflabs, Austin, TX) (Vornadran et al. 1995), was injected via sharp-electrode using a PML-100 pressure microinjector (Dagan; Minneapolis, MN), while monitoring membrane potential with the Axoclamp. Microelectrodes (as per sharp electrode, current clamp) had a resistance of 15–30 MΩ when tip-filled with 10 mM fura-PE3 and backfilled with 3 M KCl. Injections required ~3–10 200- to 300-ms pulses at 50–100 kPa to fill neurons with dye—estimated to be 50–100 µM. Neurons used for imaging had resting potentials of ~50 to ~60 mV and displayed action potentials that overshot 0 mV (evoked by 0.5- to 1-nA pulses from the amplifier). After dye injection, neurons were allowed to equilibrate for ~1 h. Imaging was performed using a Nikon TS100-F inverted microscope (Nikon; Mississauga, ON, Canada) equipped with Nikon Plan Fluor ×10 [numerical aperture (NA) = 0.5], ×20 (NA = 0.5), or ×40 (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 liquid-light guide. Excitation wavelengths were 340 and 380 nm. Between acquisitions, 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 intensified voltage was set based on the initial fluorescence intensity of the cells and maintained constant thereafter. The camera black level was set prior to an experiment such that at a gain of 1 and with no light going into the camera there was a 50:50 distribution of blue and black pixels on the display. Fluorescence intensities were sampled at 60-s intervals using regions of interests defined over the somata prior to the start of the experiment and averaged 8 frames per acquisition. The focal plane was set to approximately the middle of the neuron. The ratio of emission following 340- and 380-nm excitation (340/380) was taken to reflect free intracellular Ca²⁺ and saved for subsequent analysis. Black level determination, image acquisition, frame averaging, emitted light sampling, and ratio calculations were carried out using ImageMaster Pro.

**Reagents and drug application**

Solution exchanges were accomplished by manual perfusion using a calibrated transfer pipette to first replace the bath (tissue culture dish) solution. Drugs were applied as a small volume (<10 µl) of concentrated stock solution mixed with a larger volume of saline (~100 µl) that was initially removed from the bath then pipetted back into the bath. Care was taken to add drugs near the side of the dish and as far away as possible from the neurons. Bafilomycin A (B1793; Sigma-Aldrich), calpeptin (03–34–0051; Calbiochem, San Diego, CA) was buffered using a 10 mM HEPES; pH 7.3 with KOH. ImageMaster Pro software (version 1.49; Photon Technology International) was used to determine the calcium content. ImageMaster Pro software (version 1.49; Photon Technology International) was used to determine the calcium content.
CA), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 21857; Sigma-Aldrich), cyclopiazonic acid (CPA; C1530; Sigma-Aldrich or 239805; Calbiochem), and oligomycin A (75351; 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 current, voltage, or intracellular Ca2+. NiCl2 (N6136; Sigma-Aldrich), 1-[β-(3-[4-methoxyphenyl] propoxy)-4-methoxyphenethyl]-1H-imidazole hydrochloride (SKF-96365; 567310; Calbiochem), xanthisine (X402, Sigma-Aldrich), and xanthisine oxide (X4500, Sigma-Aldrich) were made up in distilled water. N-ethylmaleimide (NEM; E3876; Sigma-Aldrich) was dissolved as a stock in 100% ethanol.

**Analysis**

Clampfit, a program of pClamp, was used to measure current and voltage. For most of these experiments, the analysis involved comparing the average value during a steady-state baseline of 1–5 min with the average value from regions that had reached a peak for 5–30 s or remained stable for 5–10 min after the delivery of a drug. All measurements of current were normalized to cell size by dividing by whole cell capacitance (pA/pF). The current-voltage relationships and reversal potentials were determined from a difference current generated by a −90- to 0-mV ramp given before and after FCCP. To derive the difference current, the ramp current recorded prior to the addition of FCCP was subtracted from the ramp current taken at the peak of the response induced by FCCP. Conductance was derived using Ohm’s law and the current during a 200-ns step from −60 to −70 mV. The percentage change was calculated from the conductance before and after addition of FCCP.

Origin (version 7; OriginLab; Northampton, MA) was used to import and plot ImageMaster Pro files as line graphs. For intracellular Ca2+ experiments, analysis compared the steady-state value of the baseline 340/380 ratio with the ratio from regions that had reached a peak. Hill curve fits were also generated in Origin and provided the 50% effective concentration (the concentration that is required for 50% of maximal activation) as well as the Hill coefficient.

Summary data are presented as means ± SE. Statistics were performed using Instat (version 3.0; GraphPad Software, San Diego, CA). The Kolmogorov-Smirnov method was used to test data sets for normality. If the data were normal, Student’s paired or unpaired t-test was used to test for differences between two means, while a standard one-way ANOVA with Dunn’s multiple comparisons test was used to test for differences between multiple means. In cases where data were not normal, the Welch correction was applied to Student’s paired or unpaired t-test when testing for differences between two means, while a nonparametric Kruskal-Wallis ANOVA with Dunn’s multiple comparisons test was used to test for difference between multiple means. Data were considered significantly different if the P value was <0.05.

**RESULTS**

**Bag cell neurons are depolarized by the protonophore, FCCP, but not the endoplasmic reticulum Ca2+-ATPase blocker, CPA**

In the process of investigating bag cell neuron Ca2+-induced Ca2+ release, Geiger and Magoski (2008) reported that the release of mitochondrial Ca2+ by FCCP depolarized bag cell neurons. To investigate this quantitatively, the membrane potential of cultured bag cell neurons was recorded using sharp electrode current clamp. As expected, 20 μM FCCP (n = 18) caused a pronounced depolarization of ~35 mV (Fig. 1A, left). On occasion, a burst of action potentials was observed along with the depolarization. To test whether the endoplasmic reticulum had a similar effect, Ca2+ was released by blocking the Ca2+-ATPase with 20 μM CPA. Interestingly, bag cell neurons treated with CPA exhibited no change in membrane potential (n = 8; Fig. 1A, right). The depolarizing effect of FCCP was significant in comparison to CPA (Fig. 1B).

Many of the subsequent experiments were performed using whole cell electrodes instead of sharp electrodes. In whole cell mode, an ATP-containing intracellular solution is dialed into the cell via the pipette, which is not the case for sharp-electrode mode. To control for this, 20 μM FCCP was delivered to cultured bag cell neurons in current clamp under whole cell configuration (n = 8). There was no significant difference in the average change in membrane potential produced by FCCP in whole cell versus sharp-electrode (Fig. 1B).

**FCCP, but not CPA, causes an inward current**

We next examined the result of Ca2+ liberation from the mitochondrion or the endoplasmic reticulum on membrane current. Cultured bag cell neurons were whole cell voltage-clamped at −60 mV with regular intracellular saline (K+-aspartate based; 300 nM free Ca2+) in the pipette. The release of mitochondrial Ca2+ with 20 μM FCCP (n = 10) generated a slow, inward current of ~1 pA/pF (Fig. 2A). Under the same conditions, depletion of endoplasmic reticulum Ca2+ with 20 μM CPA (n = 9) failed to evoke a current of significant amplitude in comparison with FCCP (Fig. 2A, bottom). Moreover, store-interaction or differential Ca2+ handling did not appear to be involved, as pretreatment with CPA (n = 9) failed to significantly alter the amplitude of the FCCP-induced current (Fig. 2B). Also we failed to observe a response to CPA when the neurons were dialyzed with an intracellular

![FIG. 1. Targeting the mitochondria with carbonyl cyanide-4-trifluoromethoxyphenyl-hydrazone (FCCP) but not the endoplasmic reticulum with cyclopiazonic acid (CPA) depolarizes bag cell neurons. A: sharp-electrode, current-clamp recordings from 2 separate bag cell neurons show that expelling Ca2+ from the mitochondria with 20 μM FCCP induces depolarization and spiking (left), but depleting endoplasmic reticulum Ca2+ stores with 20 μM CPA does not (right). B: summary data of the mean change in membrane potential show that the effect of FCCP readily meets the level of significance when compared with CPA (Kruskal-Wallis nonparametric ANOVA; Dunn’s multiple comparisons test); however, the effect of FCCP is not significantly different when whole cell (WC), rather than sharp-electrode (SE), is employed for current-clamp recording.](http://jn.physiology.org/Downloaded from http://jn.physiology.org/ by 10.220.32.246 on April 20, 2017)
saline containing 1 μM free Ca^{2+} (change in holding current = 0.03 ± 0.02 pA/pF; n = 4; P > 0.05, one sample t-test).

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

The 20 μM of FCCP represented a concentration our laboratory (Gardam et al. 2008; Geiger and Magoski 2008) and others (Fulceri et al. 1991; Glitsch et al. 2002; Jonas et al. 1997) have used to reliably expel mitochondrial Ca^{2+}. Because FCCP collapses mitochondrial membrane potential and releases Ca^{2+} in a concentration-dependent fashion (Heytler and Prichard 1962), we expected the FCCP-induced current would have a similar concentration dependency. To test this, FCCP was applied at 30 and 300 nM and 1, 3, and 20 μM to cultured bag cell neurons, whole cell voltage-clamped at a holding potential of −60 mV (Fig. 3A). The concentration-response...
curve revealed a half-maximal effective concentration of \( \sim 1 \) 
\( \mu \text{M} \) and a lack of cooperativity, indicated by a Hill co-efficient of
\( 1.1 \) (Fig. 3B). Furthermore, the curve began to plateau at \( \sim 3 \)
\( \mu \text{M} \), placing 20 \( \mu \text{M} \) FCCP well at the top of the curve.

The time to onset of the FCCP-induced current varied from
neuron to neuron as well as between concentrations. The
current could appear as soon as 30 s but at times was delayed
for several minutes. This is apparent in both the sample current
traces displayed in Fig. 3A and in some of the examples
provided for subsequent experiments. At every concentration,
there was substantial variance in onset time with no statistical
difference overall (30 nM: 2.00 \( \pm 0.52 \) min, \( n = 5 \); 300 nM:
1.59 \( \pm 0.63 \) min, \( n = 5 \); 1 \( \mu \text{M} \): 2.82 \( \pm 0.96 \) min, \( n = 5 \); 3 \( \mu \text{M} \):
1.17 \( \pm 0.50 \) min, \( n = 5 \); 20 \( \mu \text{M} \): 2.23 \( \pm 1.00 \) min, \( n = 7 \); \( P >
0.05 \), standard ANOVA). The degree of scatter was likely due
to both the diffusion time of FCCP following the manual
addition of the drug into the bath (see METHODS) as well as the
rate at which \( \text{Ca}^{2+} \) was depleted from the mitochondria. We
have observed a similar variance in onset using \( \text{Ca}^{2+} \) imaging
and FCCP, both in the present study (see Figs. 6–9) and in
prior work (Geiger and Magoski 2008). In addition, changes to
\( \text{Ca}^{2+} \) following mitochondrial depolarization in oligodendro-
cytes and DRG neurons also present onset variability (Simpson
and Russell 1996; Werth and Thayer 1994).

**FCCP-induced current is consistent with opening of a
current channel**

To characterize the channel responsible for the FCCP
response, we examined the reversal potential and membrane
capacitance under control conditions and after the current
induced by 20 \( \mu \text{M} \) FCCP had reached peak. The protocol
involved delivery of a 200-ms step from \(-60 \) to \(-70 \) mV
followed by a 10-s ramp from \(-90 \) to 0 mV. Changes in
membrane capacitance were calcualted from the current dur-
ding the step. The difference between the current during the
ramp before and at peak was taken to be the current evoked by
FCCP. The reversal potential was derived from the point where
the difference current crossed the abscissa. Consistent with
channel opening during the FCCP-induced current, whole cell
conductance increased by over 20 times, which readily met the
level of significance compared with control (\( n = 9 \); Fig. 4A).
In normal extracellular saline (\( n = 12 \)), the current-voltage rela-
tionship was largely linear and reversed around \(-40 \) mV,
suggesting a voltage-independent nonselective cation channel
(Colquhoun et al. 1981; Hung and Magoski 2007; Partridge et al.
1994). Some cation channels are \( \text{Ca}^{2+} \)-permeable (Chesney-Mar-
chais 1985; Geiger and Magoski 2009; Magoski et al. 2000); when
\( \text{Ca}^{2+} \) was removed from the external solution (\( n = 5 \)), the current
remained linear and the reversal shifted to approximately
\(-47 \) mV, implying that \( \text{Ca}^{2+} \) passes through the channel (Fig. 4, B and
C). Moreover, decreasing intracellular \( K^{+} \) from 570 to 70 mM
(\( n = 6 \), by dialyzing neurons for 30 min with low \( K^{+} \) intracellu-
lar saline prior to FCCP, right-shifted the reversal to around
\(-20 \) mV (Fig. 4, B and C). However, the reversal potential was
not altered by raising intracellular \( \text{Cl}^{-} \) to 570 mM following
30-min dialysis with high \( \text{Cl}^{-} \) intracellular saline (\( n = 4 \); Fig. 4C).
To further confirm \( \text{Ca}^{2+} \) permeability, we examined the FCCP-
induced current at \(-60 \) mV only. Without extracellular \( \text{Ca}^{2+} \),
the amplitude of the FCCP-induced current was significantly dimin-
ished (Fig. 4D).

**FCCP-induced current depends on intracellular \( \text{Ca}^{2+} \)**

Release of mitochondrial \( \text{Ca}^{2+} \) into the cytosol appears to be
important for triggering the current, raising the possibility it is
\( \text{Ca}^{2+} \) activated. To test this, we diazylzed cultured bug cell neurons
for 10 min with intracellular solution containing either a regular (5
mM; \( n = 7 \)) or high (20 mM; \( n = 7 \)) concentration of EGTA and
recorded the FCCP-induced current in whole cell voltage-clamp at
\(-60 \) mV. If the current was dependent on intracellular \( \text{Ca}^{2+} \)
concentration, the elevation, the high-EGTA should buffer the \( \text{Ca}^{2+} \) released from
mitochondria and inhibit the response (Naraghi 1997). The FCCP-
induced current was attenuated by nearly 2/3 with a high concen-
tration of EGTA (Fig. 5A). This decrease represented a significant
reduction in the amplitude of the current compared with regular
internal (Fig. 5B). The possibility that mitochondrial \( \text{Ca}^{2+} \) 
increased membrane permeability nonspecifically by upregulating
\( \text{Ca}^{2+} \)-sensitive proteases was addressed with the \( \text{Ca}^{2+} \) protease
inhibitor, calpeptin (Mani et al. 2008; Tsujinaka et al. 1988). As
compared with DMSO-exposed neurons (\( n = 5 \)), pretreating
neurons for 20–30 min with 100 \( \mu \text{M} \) calpeptin (\( n = 4 \)) did not
alter the FCCP-induced current (Fig. 5, C and D). This concen-
tration of calpeptin has been shown to block cytosolic \( \text{Ca}^{2+} \)-
avtivated proteases in other Aplysia neurons (Khoutsorsky and
Spira 2008; Spira et al. 2001).

Thus far, the data indicated that FCCP activates a cation current
by liberating mitochondrial \( \text{Ca}^{2+} \). Prior reports from both our
laboratory and others show that FCCP as well as CPA increase
intracellular \( \text{Ca}^{2+} \) (Geiger and Magoski 2008; Jonas et al. 1997;
Kachoei et al. 2006; Magoski et al. 2000), yet there has been little
in the way of direct comparison of either kinetics or amplitude of
these responses. To examine this quantitatively, we measured
intracellular \( \text{Ca}^{2+} \) using ratiometric imaging of fura PE3-injected
cultured bug cell neurons. With external \( \text{Ca}^{2+} \) removed, 20 \( \mu \text{M} \)
FCCP (\( n = 19 \)) caused a rapid and prominent elevation in
cytosolic \( \text{Ca}^{2+} \) that persisted for several min (Fig. 6A, top).
Delivery of 20 \( \mu \text{M} \) CPA also evoked an increase in cytosolic \( \text{Ca}^{2+} \),
although this response was less than half of that observed
with FCCP and typically decayed more quickly over time (Fig.
6A, lower). Overall, the mean change in \( \text{Ca}^{2+} \) caused by FCCP
reached the level of significance when compared with CPA (Fig.
6B). We have previously shown that CPA sufficiently depletes
\( \text{Ca}^{2+} \) from the endoplasmic reticulum such that it evokes store-
operated \( \text{Ca}^{2+} \) influx (Kachoei et al. 2006). As an internal
control, extracellular \( \text{Ca}^{2+} \) was reintroduced to those neurons
that had been exposed to CPA, and in a manner typical of
store-operated \( \text{Ca}^{2+} \) influx (Nilius 2003), this increased
intracellular \( \text{Ca}^{2+} \) (mean peak change in 340/380: 0.08 \( \pm 
0.02 \); \( n = 12 \); Fig. 6A; bottom right).

**Mechanistically different mitochondrial disruptor fails to
evoke a current**

The action of FCCP on the mitochondria is to collapse the
\( H^{+} \) gradient, which in addition to releasing \( \text{Ca}^{2+} \) also uncou-
amples the respiratory chain. Despite our experiments supplying
ATP in the whole cell pipette, it was important to control for
the potential effects of FCCP on ATP synthesis. Thus we
applied the mitochondrial ATPase inhibitor, oligomycin A
(Fluharty and Sanadi 1963; Lardy et al. 1958), while either
monitoring current or intracellular \( \text{Ca}^{2+} \). In neurons whole cell
voltage-clamped at \(-60 \) mV in nASW, 20 \( \mu \text{M} \) FCCP (\( n = 6 \)}
consistently induced an inward current, while 5 µg/ml oligomycin A failed to evoke current (n = 6) or alter the FCCP-induced current (n = 6; Fig. 7A). There was no significant change in the current produced by FCCP after pretreatment with oligomycin A; however, there was a significant difference between the average amplitude of the current generated by FCCP alone and oligomycin A alone (Fig. 7B). This could reflect that specific timing of mitochondrial Ca\(^{2+}\) release is critical in activation of the current, or that oligomycin A liberates Ca\(^{2+}\) from another source. During imaging in Ca\(^{2+}\)-free external, FCCP evoked a robust increase in intracellular Ca\(^{2+}\) (n = 12), while oligomycin A caused a modest elevation, with the onset taking approximately twice as long and the peak taking about five times as long (n = 27; Fig. 7B). The average rise in Ca\(^{2+}\) observed in response to oligomycin A was significantly lower than that to FCCP (Fig. 7D); furthermore, when FCCP was added after oligomycin A had elevated Ca\(^{2+}\) (n = 10), it did not significantly affect the amplitude of the FCCP-induced Ca\(^{2+}\) increase.

Depolarization of the mitochondria has the potential to release reactive oxygen species (Galluzzi et al. 2009), which in turn could contribute to current activation. This prospect was tested by dialyzing neurons with 250 µM xanthine and 2.5 U of xanthine oxidase while recording holding current under whole cell voltage clamp at −60 mV. The effect on inward current of the reactive oxygen species generator cocktail, which readily produces superoxide (C. A. Ward, personal communication) (see also Link and Riley 1988), was a minimal change of −0.11 ± 0.11 pA/pF over the course of 20–30 min (n = 5; P > 0.05, 1-sample t-test).

**Disruptor of acidic store H\(^{+}\) transport does not induce a current**

A second consideration is that as a protonophore, FCCP has the potential to release Ca\(^{2+}\) by collapsing the H\(^{+}\) gradient of the acidic store as well as the mitochondria. The acidic store (vesicles, lysosomes) accumulates Ca\(^{2+}\) using H\(^{+}\)/Ca\(^{2+}\) ex-
change and a $H^+$ gradient maintained by a $H^+$-ATPase (Christensen 2002; Goncalves et al. 1999). Thus it is possible that acidic store $Ca^{2+}$ contributes to the FCCP-induced current. However, while 20 $\mu$M FCCP elicited an inward current in cultured bag cell neurons whole cell voltage-clamped at $-60$ mV ($n = 6$), this was not the case for 100 nM of the V-type $H^+$-ATPase inhibitor, bafilomycin A (Bowman et al. 1988) ($n = 5$; Fig. 8A). Moreover, prior expulsion of bafilomycin A-sensitive stores did not alter the FCCP-induced current ($n = 5$; Fig. 8A and C). The amplitude of the current produced by FCCP was significantly greater compared with bafilomycin A (Fig. 8C). Imaging in $Ca^{2+}$-free external showed 20 $\mu$M FCCP caused a marked elevation that peaked within minutes ($n = 13$), while bafilomycin A elicited a far more gradual increase that plateaued at an amplitude just 15% of FCCP (Fig. 8B). FCCP evoked a significantly greater peak change in 340/380 compared with bafilomycin A (Fig. 8D). Additionally, following prior liberation of $Ca^{2+}$ from acidic stores, the subsequent rise in $Ca^{2+}$ caused by FCCP was not altered ($n = 7$) (Fig. 8, B and D).

FIG. 5. Buffering intracellular $Ca^{2+}$ decreases the amplitude of the FCCP-induced current. A: whole cell, voltage-clamp recordings from two different neurons at a holding potential of $-60$ mV show that the current produced by FCCP in the presence of regular EGTA (5 mM; left) is inhibited when intracellular $Ca^{2+}$ is buffered by a high concentration of EGTA (20 mM; right) in the pipette. In both cases, the neurons are dialyzed for 10 min prior to the addition of FCCP. The delay in current onset with high-EGTA is not characteristic of this experimental condition and is found in both data sets. B: summary data show that the mean peak current density elicited by 20 $\mu$M FCCP is significantly reduced when regular internal is replaced with high-EGTA internal (2-tailed unpaired t-test). C: current traces from 2 different neurons at a holding potential of $-60$ mV demonstrate that the response to FCCP subsequent to DMSO (left) is not changed by a 20-min pretreatment with 100 $\mu$M of the protease inhibitor, calpeptin (right). D: the average peak current observed following 20 $\mu$M FCCP and normalized to cell size is not significantly impacted by calpeptin (2-tailed unpaired t-test).

FIG. 6. Both FCCP and CPA increase intracellular $Ca^{2+}$. A: ratiometric imaging of intracellular $Ca^{2+}$ in fura PE3-injected bag cell neurons. In $Ca^{2+}$-free external, 20 $\mu$M FCCP (top) depletes the mitochondria of $Ca^{2+}$ and causes an elevation in cytosolic $Ca^{2+}$ as indicated by an increase in the intensity of the 340/380 ratio. A cell exposed to 20 $\mu$M CPA (bottom), also shows rise in intracellular $Ca^{2+}$, albeit not as large as that seen with FCCP. Confirming that the endoplasmic reticulum is actually depleted by CPA, the addition of extracellular $Ca^{2+}$ (at arrow) consistently results in a secondary rise in intracellular $Ca^{2+}$; i.e., store-operated influx. B: summary data of the mean change in 340/380 show that the effect of FCCP readily meets significance compared with CPA (2-tailed unpaired t-test; Welch corrected).
Alkylation agent attenuates the effect of FCCP on both current and intracellular Ca\(^{2+}\).

Two primary ways for Ca\(^{2+}\) to leave the mitochondria following loss of organelle membrane potential are through the mitochondrial Ca\(^{2+}\) uniporter or the mitochondrial permeability transition pore (Campanella et al. 2004). If the release of Ca\(^{2+}\) from the mitochondria can be prevented by blocking the uniporter or the transition pore, then the current initiated by FCCP should also be reduced. Ruthenium red has been used to block the uniporter in both Ca\(^{2+}\) imaging and electrophysiology experiments involving mitochondrial function (Kirichok et al. 2004; Matlib et al. 1998). Cultured bag cell neurons were dialyzed for 30 min with intracellular saline containing 100 µM ruthenium red before applying 20 µM FCCP. The amplitude of the current triggered by FCCP was unaffected by ruthenium red (control: −0.574 ± 0.088 pA/pF, n = 6 vs. ruthenium red: −0.585 ± 0.049 pA/pF, n = 5; P > 0.05, 1-tailed unpaired t-test).

We also explored the role of Ca\(^{2+}\) release through the mitochondrial permeability transition pore by delivering 20 µM FCCP to cultured bag cell neurons that had been pretreated for 30 min with either 100 µM NEM or ethanol (the vehicle). As an alkylating agent, NEM can inhibit the transition pore (Costantini et al. 1996; Hunter and Haworth 1979; Petronilli et al. 1994). Exposure to NEM lowered the current amplitude, but not the time course, by half (Fig. 9A). On average, the current was significantly reduced following treatment with NEM compared with ethanol (Fig. 9B). It was essential to take into account that attenuation of the current by NEM could be due to block of mitochondrial Ca\(^{2+}\) release or the plasma membrane channel itself. To verify that NEM was directly reducing Ca\(^{2+}\) release from FCCP-sensitive stores, intracellular Ca\(^{2+}\) was monitored in cultured bag cell neurons. In an absence of extracellular Ca\(^{2+}\), the elevation in cytosolic Ca\(^{2+}\) that elicited by FCCP was significantly lowered (nearly 40%) in the presence of NEM (Fig. 9C). The mean rise in cytosolic Ca\(^{2+}\) evoked by FCCP alone to FCCP following oligomycin A pretreatment. C: summary data of the mean peak current normalized to cell size detail that the difference between the current generated by FCCP and oligomycin A readily reaches significance; however, no difference is found when comparing the current triggered by FCCP alone to FCCP following oligomycin A pretreatment. D: summary data of the mean change in 340/380 reveal that oligomycin A evokes an increase in cytosolic Ca\(^{2+}\) that is significantly less than that elicited by FCCP; as well, addition of FCCP following oligomycin A does not significantly affect the resulting Ca\(^{2+}\) increase (Kruskal-Wallis nonparametric ANOVA; Dunn’s multiple comparisons test).
In summary, NEM reduced both the FCCP-induced current and Ca\(^{2+}\) change, suggesting the current is triggered by mitochondrial Ca\(^{2+}\).

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

In addition to the evoked current, the FCCP-induced depolarization may be furthered by recruitment of voltage-sensitive channels. For example, modest depolarization can trigger a Ni\(^{2+}\)-sensitive persistent Ca\(^{2+}\) current in bag cell neurons (Tam et al. 2009). To determine if this Ca\(^{2+}\) current plays any role in the FCCP response, we first confirmed that Ni\(^{2+}\) does not block the FCCP-induced current. Cultured bag cell neurons were whole cell voltage-clamped and pretreated with 10 mM Ni\(^{2+}\) before applying 20 \(\mu\)M FCCP. There was no significant difference in the current in the presence \((n = 6)\) or absence \((n = 6)\) of Ni\(^{2+}\) (Fig. 10, A and B). A similar result was obtained using 20 \(\mu\)M of the store-operated channel blocker, SKF-96365 (Cabello and Schilling 1993; Kachoei et al. 2006) (control: \(-1.00 \pm 0.24\ \text{nA/nF}, n = 5\) vs. SKF-96365: \(-1.23 \pm 0.14\ \text{nA/nF}, n = 6\); \(P > 0.05\), 1-tailed unpaired \(t\)-test). Next, whether Ni\(^{2+}\) had any effect on the depolarization evoked by FCCP was considered in sharp electrode current clamp. The spiking that sometimes coincided with the FCCP-induced depolarization was consistently abolished following Ni\(^{2+}\) pretreatment, which is not surprising because Ca\(^{2+}\) channels are responsible for a significant portion of the rising phase of the action potential in bag cell neurons (Acosta-Urquidi and Dudek 1981) (Fig. 10C). However, pretreatment with Ni\(^{2+}\) \((n = 5)\) did not significantly alter the FCCP-induced depolarization \((n = 18;\ \text{Fig. 10, C and D})\). These observations provide evidence against the involvement of Ca\(^{2+}\) channels in the depolarization elicited by FCCP.

**D I S C U S S I O N**

To the best of our knowledge, the present study is the first to indicate that mitochondrial Ca\(^{2+}\), rather than endoplasmic reticulum Ca\(^{2+}\), preferentially gates a membrane channel. This current produces substantial depolarization in cultured bag cell neurons, appears to be mediated by a cation conductance, and is attenuated by strong buffering of intracellular Ca\(^{2+}\) or interfering with mitochondrial Ca\(^{2+}\) release. The afterdischarge of intact bag cell neurons is associated with Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Fisheter et al. 1994), which we have shown to be dependent on Ca\(^{2+}\) from the mitochondria (Geiger and Magoski 2008). Thus cation channel activation by mitochondrial Ca\(^{2+}\) may further the afterdischarge and play a role in hormone secretion responsible for reproductive behavior.

Due to the very negative voltage across the mitochondrial membrane, Ca\(^{2+}\) enters from the cytosol via the Ca\(^{2+}\)
unipporter; FCCP takes the voltage to zero and causes Ca\(^{2+}\) to exit (Heytler and Prichard 1962; Moore 1971). Invertebrate examples include FCCP-induced depolarization or Ca\(^{2+}\) release from mitochondria isolated from flatworm or squid giant axon (Brinley et al. 1977; Mercer et al. 1999). Regarding Ca\(^{2+}\) handling, the unipporter is a ruthenium red-sensitive, voltage-independent ion channel in the inner membrane that could allow for Ca\(^{2+}\) efflux from depolarized mitochondria (Kirichok et al. 2004; Moore 1971). However, this channel shows extreme inward rectification near 0 mV, and efflux would be eliminated on depolarization. Hence it is not surprising that ruthenium red was ineffective at blocking the FCCP-induced current in bag cell neurons. Alternatively, Ca\(^{2+}\) could leave through the mitochondrial permeability transition pore, an inner membrane, voltage-dependent mega-channel opened by mitochondrial depolarization and blocked by NEM (Hunter and Haworth 1979). The actions of NEM on the transition pore are well characterized and reasonably specific; however, the drug is capable of altering sulfhydryl groups on a number of proteins (Costantini et al. 1996; Petronilli et al. 1994). Nevertheless, consistent with mitochondrial Ca\(^{2+}\) being the trigger, high intracellular EGTA reduces the FCCP current, while NEM lessens both the Ca\(^{2+}\) elevation and current evoked by FCCP. Furthermore NEM fails to alter the current opened by Ca\(^{2+}\) entry following a train of depolarizing stimuli (the present study) as well as the voltage-gated Ca\(^{2+}\) current itself (Hickey 2009), suggesting that akylation does not generally inhibit plasma membrane channels in bag cell neurons.

Because FCCP affects mitochondrial function, lowered ATP levels, rather than Ca\(^{2+}\) release, could influence the current. Oligomycin A blocks the mitochondrial ATP synthetase, which, like FCCP, would eliminate mitochondrial ATP production (Fluharty and Sanadi 1963; Lardy et al. 1958). It appears that glycolysis is able to provide sufficient levels of ATP in the short term despite reduced oxygen consumption in the face of mitochondrial poisoning (Land et al. 1999). The source of the oligomycin A-induced Ca\(^{2+}\) elevation in bag cell neurons may not be mitochondrial. Reduced ATP levels could result in Ca\(^{2+}\) loss from the endoplasmic reticulum or acidic stores, which depend on energy for Ca\(^{2+}\) uptake. However, our laboratory has reported that FCCP does not deplete Ca\(^{2+}\) from the endoplasmic reticulum (Geiger and Magoski 2008). Regardless of origin, Ca\(^{2+}\) liberated by oligomycin A does not evoke a current in bag cell neurons, substantiating the assertion that the FCCP-induced current is not due to changes in ATP. Additionally, we recorded the current using whole cell and ATP in the pipette.

**FIG. 9.** An alkylation agent attenuates both the rise in intracelluar Ca\(^{2+}\) and the current induced by FCCP. A: whole cell, voltage-clamp recordings at −60 mV, in separate neurons bathed in nASW, demonstrate a notable reduction in the amplitude of the FCCP-induced current following a 30-min pretreatment with 100 μM N-ethylmaleimide (NEM; right) but not the vehicle, ethanol (EtOH; left). B: summary data of mean peak current normalized to cell size indicate that the current evoked by 20 μM FCCP is significantly decreased subsequent to treatment with NEM versus ethanol (2-tailed unpaired t-test). C: monitoring intracellular Ca\(^{2+}\) with ratiometric imaging in Ca\(^{2+}\)-free external shows that 20 μM FCCP causes a prominent rise in cytosolic Ca\(^{2+}\) with ethanol (left); however, this effect is greatly diminished following a 30-min treatment with NEM (right). Note that application of ethanol or NEM alone does not alter intracellular Ca\(^{2+}\). D: summary data show that the mean change in 340/380 evoked by FCCP is significantly less following NEM than after ethanol (2-tailed unpaired t-test; Welch corrected). E: current traces under voltage clamp at −60 mV from different neurons. Delivery (at bar) of a 5-Hz, 1-min train of 75-ms steps to +10 mV elicits an inward current. Compared with ethanol exposure (left), the magnitude of the train-evoked current subsequent to NEM (right) is similar. Differences in time-course following the peak are observed in both data sets and similar to our prior observations (see Hung and Magoski 2007). F: summary of average peak train-evoked current normalized to capacitance. This activity-dependent current is not significantly different when ethanol is compared with NEM (2-tailed unpaired t-test).
FCCP theoretically increases the permeability of H⁺ across all membranes, potentially allowing H⁺ to contribute to resting potential (Bashford et al. 1985). However, this would not be sensitive to intracellular Ca²⁺ buffering or changes in extracellular Ca²⁺ and intracellular K⁺—which is the case for the FCCP-induced current. Moreover, the H⁺ equilibrium potential under our conditions is −30 mV and too positive with respect to the reversal potential of the current. Nevertheless, we cannot assume that the measured reversal potential is dependent solely on a single type of channel. Additional currents (mediated by H⁺ channels, exchangers, or ionophores) could be activated by FCCP and render the measured reversal potential a sum of reversal potentials, which is merely dominated by the Ca²⁺-activated cation channel we have characterized (see following text). FCCP could lower intracellular pH by drawing H⁺ out of acidic stores (Park et al. 2002; Werth and Thayer 1994). Yet a drop of merely half a pH unit would render the H⁺ equilibrium potential too negative at −60 mV. Acidification could activate pH-sensitive cation channels, but unlike the FCCP-induced current, such channels are blocked by ruthenium red (Garcia-Hirschfeld et al. 1995; Zeilhofer et al. 1996). Also the lack of an effect of bafilomycin A, which itself could acidify the cytosol, points away from H⁺ as a gating factor. Finally, FCCP and related protonophores fail to depolarize or produce a current in hamster kidney, RBL-1, and chromaffin cells (Bashford et al. 1985; Giovannucci et al. 1999; Glitsch et al. 2002; Hernandez-Guijo et al. 2001) as well as crayfish motor and hippocampal neurons (Partridge and Valenzuela 1999; Tang and Zucker 1997). If FCCP generally caused depolarization, this would manifest in all cell types.

The FCCP-induced current is mainly voltage independent, accompanied by an increase in conductance, and presents a reversal potential of −40 mV, indicative of opening a nonselective cation channel. A reversal potential between −40 and +20 mV is characteristic of a varying degree of cation selectivity and no clear preference (Colquhoun et al. 1981; Kass et al. 1978; Partridge and Swandulla 1987; Partridge et al. 1994).

Without external Ca²⁺, the FCCP-induced current is diminished, and the reversal shifts to the left, implying Ca²⁺-passage. Similarly, dialysis with a low K⁺ intracellular saline, such that the K⁺ equilibrium potential changes from around −100 mV to close to −50 mV, shifts the reversal to approximately −20 mV. However, Cl⁻ does not seem to play a role given that the current is unaffected by moving the Cl⁻ equilibrium potential from −50 mV to near 0 mV after dialyzing with high Cl⁻ intracellular saline.

Because a rise in intracellular Ca²⁺ appears to elicit the current, one or more bag cell neuron Ca²⁺-activated channels may be responsible. The voltage-dependent cation channel characterized by Wilson et al. (1996) and Geiger et al. (2009) is an unlikely candidate given that it reverses well above 0 mV, although it may contribute to the depolarization through secondary activation. A second remote prospect is the voltage-independent cation channel that reverses around −15 mV and is opened by flufenamic acid-mediated Ca²⁺ release (Gardam et al. 2008). The most likely possibility is the voltage-independent cation channel reported by Hung and Magoski (2007) that is triggered by Ca²⁺ influx, reverses near −40 mV, and is not blocked by Ni²⁺ or SKF-96365 (Tam et al. 2009). In the present study, a version of this current was evoked by a train of depolarizing stimuli and shown to be insensitive to NEM.

Where the present study suggests a link between mitochondrial Ca²⁺ and channel activation, other reports point to metabolic or Ca²⁺ buffering roles for mitochondria in regulating plasma membrane channels. For example, FCCP generates an outward current in hippocampal and locus ceruleus neurons (Hyllienmark and Brismar 1996; Murai et al. 1997; Nowicky and Duchen 1998). Those studies suggest that a reduction in ATP both opens K⁰ ATP channels and, through a lessening of the Ca²⁺ ATPase, leads to a leak of endoplasmic reticulum Ca²⁺ that in turn gates Ca²⁺-activated K⁺ channels. Furthermore, store-operated channels in Chinese hamster ovary and Jurkat T-cells, as well as IP₃ receptors in HeLa cells, are inhibited by mitochondrial depolarization but not by transition pore block.
In a number of neurons, endoplasmic reticulum \( \text{Ca}^{2+} \) either evokes or potentiates voltage-independent cation channels. \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \) release enhances depolarizing afterpotentials in supraoptic and hippocampal neurons (Li and Hatton 2003; Prawitt et al. 2003; Strubing et al. 2001; Zitt et al. 1997). In expression systems (Launay et al. 2002; Liu and Liman 1999) and turns on certain transient receptor potential channels will open membrane channels; rather, mitochondrial \( \text{Ca}^{2+} \) appears to be vital.

However, gating of such currents by mitochondrial \( \text{Ca}^{2+} \) has not been reported until now. Cultured bag cell neurons are both quite resistive and present a sizeable membrane surface area, resulting in a large membrane time constant. Thus once the current has depolarized the neuron, it may be lengthened in part by the time constant slowing recovery of the membrane potential. Because bag cell neurons release mitochondrial \( \text{Ca}^{2+} \) during prolonged action potential firing (Fisher et al. 1994; Geiger and Magoski 2008), the cation channel could contribute to the depolarization necessary for the afterdischarge.

Why does endoplasmic reticulum \( \text{Ca}^{2+} \) fail to open channels? Potentially, the reduced magnitude, rate, or duration of the \( \text{Ca}^{2+} \) rise elicited by CPA, compared with FCCP, limits the ability of endoplasmic reticulum \( \text{Ca}^{2+} \) to trigger current. That stated, the persistence of the FCCP response at low doses or in the face of a reduced \( \text{Ca}^{2+} \) response under NEM suggests that the size of the \( \text{Ca}^{2+} \) signal is less of a factor, i.e., a small amount of mitochondrial \( \text{Ca}^{2+} \) still evokes a current. Differential distribution may give mitochondrial \( \text{Ca}^{2+} \) preferential physical access to membrane channels. For example, in oligodendrocytes, mitochondria are localized to areas of \( \text{Ca}^{2+} \) signal amplification during wave propagation (Simpson and Russell 1996). Endoplasmic reticulum is sometimes more associated with the nucleus, while mitochondria are evenly distributed (Palade 1955; Verkhratsky 2005). Accordingly, dye staining of bag cell neurons shows mitochondria are present throughout the soma and neurites (White and Kaczmarek 1997). Alternatively, the channel could be gated by an intermediate, yet mobile \( \text{Ca}^{2+} \) sensor, such as calmodulin. Both Wood et al. (1980) and Pardue et al. (1981) provide evidence that calmodulin tightly associates with mitochondria. Our laboratory has established that both calmodulin and various enzymes can closely associate with channels in bag cell neurons (Gardam and Magoski 2009; Lupinsky and Magoski 2006; Magoski 2004; Magoski and Kaczmarek 2005). If the \( \text{Ca}^{2+} \) sensor favors association with the mitochondria, as opposed to the endoplasmic reticulum, then mitochondrial \( \text{Ca}^{2+} \) could be privileged for channel activation. In fact, this may be key to why simply increasing \( \text{Ca}^{2+} \) with CPA or bafloimycin does not have an effect. Finally, along with opening the cation channel, a secondary \( \text{Ca}^{2+} \)-dependent process could synergize with mechanisms already discussed (lengthy time constant, voltage-dependent channels) to extend or further the depolarization.

To summarize, \( \text{Ca}^{2+} \) from mitochondria opens a cation channel that profoundly impacts neuronal activity. This regulation appears unique in so much that it is not recapitulated by endoplasmic reticulum \( \text{Ca}^{2+} \). Given the role of mitochondrial permeability in apoptosis and excitotoxicity (Campanella et al. 2004; Galluzzi et al. 2009), gating of a depolarizing current by this \( \text{Ca}^{2+} \) source also has implications for pathophysiology.

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