Ca\(^{2+}\) -induced uncoupling of Aplysia bag cell neurons

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ELECTRICAL COUPLING via gap junctions is found in many cell types (Dermietzel and Spray 1993), including neurons, where it synchronizes activity (Bennett and Zukin 2004; Sohl et al. 2005; Spray and Bennett 1985). Like their chemical counterparts, electrical synapses are subjected to modulation by various second messengers and effectors (Lampe and Lau 2004; Neyton and Trautmann 1986; Peracchia 2004). One of the earliest described forms of regulation is the inhibition of gap junctions by increased intracellular Ca\(^{2+}\) (Spray and Bennett 1985), which was first reported by Loewenstein et al. (1967) in salivary glands of the midge Chironomus. Subsequently, this has been investigated in many nonneuronal and neuronal cells by intracellular injection of Ca\(^{2+}\) (De Mello 1975; Schirmacher et al. 1996) or liberating Ca\(^{2+}\) with either pharmacological agents (Baux et al. 1978; Dahl and Isenberg 1980; Mus-

Intracellular Ca\(^{2+}\) can modulate both vertebrate (Rao et al. 1987) and invertebrate (Baux et al. 1978; Pereda et al. 1998) neuronal coupling and likely acts through the activation of signal transduction pathways such as calmodulin (CaM) (Peracchia 2004) and CaM-kinases (Pereda et al. 1998). Gap junction proteins bind CaM directly, and some are phosphorylated by CaM-kinase II (Alev et al. 2008; Burr et al. 2005; Peracchia et al. 2000; Van Eldik et al. 1985). In the present study, we used the bag cell neurons of Aplysia to explore the Ca\(^{2+}\)-dependent modulation of electrical synapses.

The bag cell neurons of the marine mollusk Aplysia californica are neuroendocrine cells located at the rostral end of the abdominal ganglion in two clusters of 200–400 neurons. They initiate reproduction through the secretion of egg-laying hormone during a lengthy and synchronous afterdischarge (Kaczmarek et al. 1978; Kupfermann and Kandel 1970; Pinsker and Dudek 1977; Rothman et al. 1983). This burst presents as a fast phase of firing (~5 Hz for ~1 min) followed by a slow phase of firing (~1 Hz for ~30 min). Synchrony is achieved through electrical coupling, both between neurons in a cluster as well as from one cluster to the other (Blankenship and Haskins 1979; Brown et al. 1989; Kaczmarek et al. 1979). The afterdischarge is accompanied by an increase in intracellular Ca\(^{2+}\) due to influx and release (Fisher et al. 1994; Michel and Wayne 2002). Furthermore, protein kinase C (PKC) is activated shortly after the onset of firing (Conn et al. 1989a; Wayne et al. 1999) and serves to potentiate Ca\(^{2+}\) channels (DeRiemer et al. 1985; Knox et al. 1992; Tam et al. 2009).

Here we performed whole cell recording on paired cultured bag cell neurons to show that Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels, or Ca\(^{2+}\) liberated from the mitochondria, attenuates electrical coupling in a CaM-kinase-dependent fashion. Triggering PKC coincident with Ca\(^{2+}\) entry inhibits electrical synapses to an even greater extent, although never to the point of decoupling. In the intact cluster, this may bolster excitability by decreasing the net leakiness of a given neuron. Ca\(^{2+}\) influx also decreases the amplitude and duration of electrotonic transmission, which may improve synchrony by preventing lengthy responses and out-of-phase postsynaptic action potentials. Hence modulation of electrical coupling may help maintain and augment the release of reproductive hormone.

MATERIALS AND METHODS

Animals and cell culture. Adult A. californica (a hermaphrodite) weighing 150–500 g were obtained from Marinus (Long Beach, CA), housed in an ~300-liter aquarium containing continuously circulating, aerated artificial seawater (ASW) (Instant Ocean; Aquarium Systems, Mentor, OH) at 14–16°C on a 12:12-h light-dark cycle, and...
fed romaine lettuce five times per week. Experiments were approved by the Queen’s University Animal Care Committee (protocols Magoski-100323 and Magoski-100845).

For primary culture of isolated bag cell neurons, animals were anesthetized by an injection of isotonic MgCl₂ (50% of body wt) and the abdominal ganglion was removed and treated with dispase II (13.3 mg/ml; 165859; Roche Diagnostics, Indianapolis, IN) dissolved in tissue culture (tc)ASW (composition in mM: 460 NaCl, 10.4 KCl, 11 CaCl₂, 55 MgCl₂, and 15 HEPES, with 1 mg/ml glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, pH 7.8 with NaOH) for 18 mg/l at 22°C. The ganglion was then rinsed in tcASW for 1 h, and the bag cell neuron clusters were dissected from their surrounding connective tissue. With a fire-polished glass Pasteur pipette, neurons were dissociated by gentle trituration and dispersed in tcASW onto 35 × 10-mm polylysine tissue culture dishes (353001; Falcon Becton-Dickinson, Franklin Lakes, NJ). Neurons were paired by moving tcASW in or out of the pipette to push or pull a free neuron into contact with a neuron already adhered to the dish. Neurons were plated with either their somata (soma-soma) or primary neurites (neurite-neurite) in contact. For Ca²⁺ imaging (see below), single neurons were plated. Cultures were maintained in a 14°C incubator in tcASW and used for experimentation within 2–4 days. Salts were obtained from Fisher (Ottawa, ON, Canada), ICN (Aurora, OH), or Sigma-Aldrich (Oakville, ON, Canada).

Whole cell voltage- and current-clamp recording. Recordings of membrane potential or current were made from cultured bag cell neurons with two EPC-8 amplifiers (HEKA Electronics, Mahone Bay, NS, Canada) and the tight-seal whole cell method. Microelectrodes were pulled from 1.5-mm-external diameter/1.12-mm-internal diameter borosilicate glass capillaries (TW150F-4; World Precision Instruments, Sarasota, FL) and had a resistance of 1–2 MΩ when filled with our standard intracellular saline [composition in mM: 500 K⁺ – aspartate, 70 KCl, 1.25 MgCl₂, 10 HEPES, 11 glucose, 10 glutathione, 5 EGTA, 5 ATP (grade 2, disodium salt; A-3577, Sigma-Aldrich), and 0.1 GTP (type 3, disodium salt; G-8877, Sigma-Aldrich); pH 7.3 with KOH]. The free Ca²⁺ concentration was set at 300 nM by adding 3.75 mM CaCl₂, as calculated by WebbMaxC (http://web.stanford.edu/~capton/webmaxcs.htm). In two separate sets of experiments, an intracellular saline with either 0 mM EGTA and no added Ca²⁺ or 20 mM EGTA and 35 nM free Ca²⁺ (4.14 mM added CaCl₂) was used. Neuronal pairs were usually dialyzed for 30 min under voltage clamp at −60 mV before experimentation. Our lab has found that this length of time is necessary to ensure that, as much as possible, the intracellular ions are exchanged with those of the pipette (White and Magoski 2012). In turn, this avoided any potential run-up or rundown of junctional current that could arise from differences or ongoing changes in intracellular ion concentration.

Recordings were performed in normal (n)ASW (composition as per tcASW, but with glucose and antibiotics omitted). The standard intracellular saline had a calculated liquid junction potential of 15 mV vs. ASW, which was corrected by off-line subtraction. For a subset of experiments, some pairs were bathed in Ca²⁺-free ASW (composition as per nASW but with no added CaCl₂, 66 mM MgCl₂, and 0.5 mM EGTA). Pipette junction potentials were nulled immediately before seal formation. After membrane rupture, pipette and neuronal capacitance were canceled and the series resistance (3–5 MΩ) was compensated to 70–80% and monitored throughout the experiment. Input signals were filtered at 1 kHz (for current) and 3 kHz (for voltage) by the EPC-8 Bessel filter and sampled at 2 kHz with an IBM-compatible personal computer, a Digidata 1322A analog-to-digital converter ( Molecular Devices; Sunnyvale, CA), and the Clampex acquisition program of pCLAMP 8.1 (Molecular Devices). Clampex was used to control the membrane potential under voltage clamp and to inject current steps under current clamp; in addition, neurons were manually current-clamped to set membrane potentials by delivering constant bias current with the EPC-8 V-hold. Most data presented here are for junctional current, which is the current provided by the amplifier to maintain the voltage clamp of the postsynaptic neuron at −60 mV, after creation of a voltage difference across the gap junction by a voltage step (to −90 mV) to the presynaptic neuron. Ca²⁺ imaging. For Ca²⁺ imaging, single neurons were dialyzed with standard intracellular saline supplemented with 1 mM fura-PE3 (K⁺ salt; 0110, Teffabs, Austin, TX) (Vorndran et al. 1995). Imaging was performed with a TS100-F inverted microscope (Nikon) equipped with a Nikon Plan Fluor ×20 (numerical aperture = 0.5) objective. The light source was a 75-W Xe arc lamp and a multiwavelength 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 and delivered through a computer-controlled shutter with a Photon Technology International computer interface and EasyRatio Pro software 1.10 (Photon Technology International). During acquisition, the shutter remained open and emitted light passed through a 400-nm long-pass dichroic mirror and a 510/40-nm emission barrier filter before being detected by a Cool SNAP HQ2 charge-coupled device camera (Photometrics, Tucson, AZ). The camera gain was maximized and the exposure time set at 1 s for both 340- and 380-nm excitation wavelengths. Fluorescence intensities were sampled at 0.5 Hz using regions of interest measured over the somata at approximately the midpoint of the vertical focal plane and one-half to three-quarters of the cell diameter, then averaged eight frames per acquisition. The ratio of the emission after 340- and 380-nm excitation (340/380) was taken to reflect free intracellular Ca²⁺ (Grynkiewicz et al. 1985) and saved for subsequent analysis. Image acquisition, emitted light sampling, and ratio calculations were performed with EasyRatio Pro.

Reagents and drug application. Solution changes were accomplished by using a calibrated transfer pipette to exchange the bath (tissue culture dish) solution. Drugs were introduced by initially removing a small volume (~75 μL) of saline from the bath, combining that with an even smaller volume (~10 μL) of drug stock solution, and then reintroducing that mixture back into the bath. Care was taken to pipette near the side of the dish and as far away as possible from the neurons. Phorbol 12-myristate 13-acetate (PMA; P-8139, Sigma-Aldrich), cyclopiazonic acid (CPA; C-1530, Sigma-Aldrich), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP; 21857, Sigma-Aldrich), KN-62 (I-2142, Sigma-Aldrich), KN-92 (422709, Calbiochem/EMD Millipore, Billerica, MA), KN-93 (422708, Calbiochem), and ryanodine (559276, Calbiochem) were dissolved as stocks in dimethyl sulfoxide (DMSO; BP231-1, Fisher). The maximal final concentration of DMSO ranged from 0.05% to 0.5% (vol/vol), which in control experiments both here and in prior work from our laboratory had no effect on holding current, membrane conductance, or junctional current (Dargaei et al. 2014; Hickey et al. 2010, 2013; Kachoei et al. 2006; Tam et al. 2011).

Data analysis. Most analysis involved cell pairs designated as neuron 1 and neuron 2, based on being recorded with the left and right amplifier, with membrane potentials and membrane currents specified as V1, V2, I1, and I2, respectively. The Clampfit (8.1 or 10.2) analysis program of pCLAMP was used to determine the amplitude and time course of membrane current or voltage. For current, vertical cursors were placed along the baseline, both prior to any voltage step and when the junctional current had reached steady-state at 20–30 ms before the end of the step to −90 mV. Some measurements were made by using the horizontal cursor as a guide to effectively adjacent average the two regions and find average current levels; the difference between the two was then taken as the current change. Measurements were also made by using two vertical cursors placed to span the 10 ms immediately prior to the current change evoked by the step, while an additional two cursors were positioned 20–30 ms before the end of the junctional current and just before the end of the junctional current, respectively. Clampfit then calculated the average current between each pair of cursors, and the difference between the averages was taken as the current change. The percent
change in junctional current following Ca\(^{2+}\) influx due to stimulation or the addition of a drug was calculated by comparing the subsequent current to control. The area of the electrotonic potential (ETP) was measured by placing cursors at the onset of the ETP and at the steady-state voltage after the ETP, i.e., when the postsynaptic membrane potential had fully returned to the pre-ETP baseline. The difference between the two cursors was taken as the region of the ETP, under which the ETP was measured (Carlin et al. 2000).

The persistent calcium current \(I_{Ca}\) is a high-voltage-activated (L-like) current, described by

\[
\frac{dy}{dt} = \frac{y_s(V) - y}{\tau_y}, \quad y_s = \frac{1}{1 + \exp(V - \theta_y/z)
\]

where \(y_{Ca} = 4 \text{ ms/cm}^2\) is its conductance strength, \(E_{Ca} = 60 \text{ mV}\) is its reversal potential, and \(y\) gates its activation. The steady-state value of \(y\) is given by \(y_s\), where \(\theta_y = -10 \text{ mV}\) is the voltage at half-activation and \(z = -6 \text{ mV}\) determines the slope of the function (Carlin et al. 2000).

Synaptic current \(I_{Syn}\) is comprised of acetylcholine receptor-mediated current \(I_{ACH}\) and gap junction-mediated current \(I_{gapp}\). The former is given by \(I_{ACH} = g_{ACH}(V - E_{ACH})\), where \(g_{ACH} = 0.05 \text{ ms/cm}^2\) is its conductance strength, \(E_{ACH} = 0 \text{ mV}\) is its reversal potential, and receptor activation is described by

\[
dx_{ACH}/dt = -x_{ACH}/\tau_{ACH} + \delta(t - t_i)
\]

where \(\tau_{ACH} = 25 \text{ ms}\) is the time constant of decay, \(\delta\) is the Dirac delta function, and \(t_i\) is the time of firing of a presynaptic neuron. For the gap junction-mediated current \(I_{gapp}\), the coupling between neurons followed a “nearest neighbor” approach:

\[
I_{gapp} = \frac{g_{gap}(V_{1} + V_{2}) - 2(V_{1} + V_{2})}{V_{1} - V_{2}} - 1
\]

where \(j = 2:99\) is the index of an individual neuron and \(g_{gap}\) is its conductance strength (Ermentrout et al. 2004). For the special cases of \(j = 1\) and \(j = 100\), the neurons were only coupled to one other cell. Presynaptic spikes were simulated by periodic spike trains at 20 Hz, provided to neurons 35–64.

RESULTS

Cultured pairs of bag cell neurons show electrical coupling. Previous morphological and electrophysiological studies of bag cell neurons in the intact cluster showed that the bag cell neurons are electrically coupled (Blankenship and Haskins 1979; Brown et al. 1989; Kaczmarek et al. 1979). To study these synapses under more controlled conditions, we dissociated bag cell neurons and brought them into contact at either the somata (Fig. 1A) or the neurites (Fig. 1B). After 2–4 days in culture, ~80% of pairs usually exhibited electrical coupling. Whole cell recording, in either voltage or current clamp, was used in all cases. Unless stated otherwise, recordings were made in nASW as the extracellular solution and a K\(^{+}\)Asp-based intracellular saline in the pipette (see MATERIALS and METHODS for details). Cells were designated as neuron 1 and neuron 2, with neuron 1 always as the presynaptic cell and neuron 2 the postsynaptic cell, the rationale being that electrical coupling between cultured bag cell neurons is non-rectifying and has the same magnitude when either neuron 1 or neuron 2 serves in a presynaptic role (Dargaei et al. 2014).

To confirm the existence of electrical synapses between cultured bag cell neurons, a 1-nA, 5-s depolarizing current was injected into one neuron under current clamp, which usually elicited a train of nonaccommodating action potentials over the duration of the stimulus (\(n = 25\)) (Fig. 1C). Both the steady-state depolarization and action potentials in the stimulated neuron generated depolarization and ETPs in the second neuron. In some cases, electrotonic transmission was able to recruit the coupled cell and provoke postsynaptic action potentials (\(n = 9\) of 25).
whether Ca$^{2+}$ and Wayne 2002). Ca$^{2+}$ however, it is relatively rare to find cases of Ca$^{2+}$
1982) and neuronal (Baux et al. 1978; Rao et al. 1987) cells; 
tions in both nonneuronal (Loewenstein et al. 1967; Spray et al.
corresponds to current flowing across the electrical synapse. [52x120]
second neuron was maintained at 
60 mV, the outward current 
60 mV, induced current 
both neurons. Figure 1
D
in both neurons. Figure 1
increased intracellular Ca$^{2+}$ inhibits the electrical synapse. 
Prior work indicated that the afterdischarge resulted in an 
increase in intracellular Ca$^{2+}$ due to both voltage-gated influx 
and release from intracellular stores (Fisher et al. 1994; Michel 
and Wayne 2002). Ca$^{2+}$ has been shown to inhibit gap junc-
tions in both nonneuronal (Loewenstein et al. 1967; Spray et al. 1982) 
and neuronal (Baux et al. 1978; Rao et al. 1987) cells; however, 
it is relatively rare to find cases of Ca$^{2+}$ channels 
being the source of the rise in Ca$^{2+}$. Thus we examined 
whether Ca$^{2+}$ influx, produced by a train-stimulus of 75-ms 
pulses from $-60$ to 0 mV at 5 Hz for 1 min and then 1 Hz for 
5 min, which mimics the fast and early slow phases of the 
afterdischarge, respectively, suppresses junctional current. 
Electrically coupled cultured bag cell neurons were volt-
age-clamped, and the junctional current was measured in the 
postsynaptic neuron (Fig. 2A, inset) after a 30-min period to 
allow for full dialysis. When neurons were not stimulated 
($n = 6$), junctional current showed little change over time 
(Fig. 2A). However, delivering the combined train-stimulus 
to both neurons after 30 min of dialysis caused an $\sim 30\%$ 
decrease in junctional current (Fig. 2B) ($n = 7$). To confirm 
that Ca$^{2+}$ was inhibiting electrical communication, extra-
cellular Ca$^{2+}$ was removed by substituting with Mg$^{2+}$. With 
no Ca$^{2+}$ in the bath ($n = 7$), the train-stimulus failed to alter 
the junctional current (Fig. 2C). The difference in the 
percent change in junctional current for control vs. Ca$^{2+}$- 
free conditions was not significant; however, the change 
seen after Ca$^{2+}$ influx was significantly different from both 
control and Ca$^{2+}$-free conditions (Fig. 2D).
We also examined the time course of the change to intra-
cellular Ca$^{2+}$ in response to the 5-Hz, 1-min then 1-Hz, 5-min 
stimulus in fura-PE3-loaded cultured bag cell neurons from a 
holding potential of $-60$ mV under voltage clamp. There was 
an immediate and robust Ca$^{2+}$ elevation with delivery of the 
5-Hz train, followed by a less substantial plateau during the 
1-min train (Fig. 2, E and F). Based on a comparison with prior 
Ca$^{2+}$ measurements made by Fisher et al. (1994) in bag cell 
neurons with Ca$^{2+}$-sensitive electrodes, we estimated that the 
$\sim 0.4$-unit (340/380) change during the 5-Hz stimulus and the 
0.175 unit change during 1-Hz stimulus translated roughly to 
deltas of 400 nM and 200 nM Ca$^{2+}$, respectively, rendering a 
shift in Ca$^{2+}$ from a baseline of $\sim 200–300$ nM to a peak of 
$\sim 600–700$ nM and a plateau of $\sim 400–500$ nM.
Synchronous stimulation is most effective at inhibiting electrical coupling. Closure of gap junctions has been reported to require the increase of cytosolic Ca\(^{2+}\) to a specific threshold (Rose and Loewenstein 1976). Therefore we examined whether a Ca\(^{2+}\) rise in only one cell from a pair of cultured bag cell neurons was sufficient to inhibit the electrical synapse. Subsequent to a 30-min dialysis under voltage clamp, junctional current was induced (Fig. 3A, inset). As a control, the train-stimulus was simultaneously delivered to both neurons (n = 5), which resulted in an ~25% decrease in coupling that

**Fig. 2. Elevation of intracellular Ca\(^{2+}\) decreases junctional current.** A: neurons are whole cell voltage-clamped at −60 mV in normal artificial seawater (nASW) with standard K\(^+\)-Asp-based intracellular saline for 30 min to ensure complete dialysis. The voltage change in neuron 1 produces membrane current in neuron 1 (I1) and junctional current in neuron 2 (I2). Inset shows the step voltage to assay junctional current. Scale bars apply to both traces. A dashed line extending from the end of the initial junctional current (I2, left) is placed at the estimated steady-state level to aid in comparison with the subsequent trace (right). B: Ca\(^{2+}\) influx, produced by simultaneously delivering to both cells (stim) a train of 75-ms pulses from −60 to 0 mV at 5 Hz for 1 min followed by 1 Hz for 5 min decreases the junctional current (after) compared with the initial response (before). The altered junctional current is recorded immediately after stimulation. For B and C only the junctional current (I2) is presented. Inset shows a pair of neurons with stimulation to both pipettes. Scale bars apply to both traces. C: in a Ca\(^{2+}\)-free extracellular saline, where Mg\(^{2+}\) is substituted for Ca\(^{2+}\), the train-stimulus does not change the junctional current. Scale bars apply to both traces. D: summary graph showing that the % change in junctional current significantly decreases after stimulation in the presence of extracellular Ca\(^{2+}\) vs. both the unstimulated control and neurons stimulated in Ca\(^{2+}\)-free solution (F\(_{2,17}\) = 11.83, P < 0.05, ANOVA; Tukey-Kramer multiple-comparisons test). For this and subsequent bar graphs, the n value (typically no. of pairs of cultured bag cell neurons) is shown in parentheses within or just above or below individual bars. E: measurement of intracellular Ca\(^{2+}\) in a single cultured bag cell neuron filled with fura-PE3. The neuron is voltage-clamped at a holding potential (HP) of −60 mV and, after 15 min of dialysis to dye-load, the stimulation train is delivered (as per B and C). The change in the ratio of the emission following 340 and 380 nm excitation (340/380) reflects elevated free intracellular Ca\(^{2+}\). During the 5-Hz, 1-min stimulus, the Ca\(^{2+}\) presents a large-amplitude component that declines to a stable elevation over the course of the 1-Hz, 5-min stimulus. F: group data comparing the change in 340/380, taken from prestimulus baseline to the peak of the response at 5 Hz and the plateau at 1 Hz, show a significant difference between the 2 frequencies (unpaired Student’s t-test).

**Synchronous stimulation is most effective at inhibiting electrical coupling.** Closure of gap junctions has been reported to require the increase of cytosolic Ca\(^{2+}\) to a specific threshold (Rose and Loewenstein 1976). Therefore we examined whether a Ca\(^{2+}\) rise in only one cell from a pair of cultured bag cell neurons was sufficient to inhibit the electrical synapse. Subsequent to a 30-min dialysis under voltage clamp, junctional current was induced (Fig. 3A, inset). As a control, the train-stimulus was simultaneously delivered to both neurons (n = 5), which resulted in an ~25% decrease in coupling that
lasted for up to 30 min (Fig. 3A). However, giving the same train-stimulus only to neuron 1, while keeping neuron 2 at −60 mV throughout (n = 9), caused a smaller inhibition of ~10% that decayed to no change by 30 min (Fig. 3B). When the change in junctional current following stimulation of both neurons was compared to that produced by stimulating just one neuron, the level of uncoupling was found to be significantly greater immediately after Ca2+ influx and 4 min later (Fig. 3C).

Depletion of mitochondrial Ca2+ attenuates junctional current. Fisher et al. (1994) demonstrated that Ca2+-induced Ca2+ release occurred from intracellular stores during the slow phase of the afterdischarge, and this required an interaction between mitochondria and endoplasmic reticulum (Geiger and Magoski 2008; Groten et al. 2013). As such, EGTA and Ca2+ were removed from the pipette solution (to avoid influencing store-derived Ca2+) and the impact of Ca2+ liberation from either the endoplasmic reticulum or mitochondria on electrical coupling was examined. After a 30-min dialysis, junctional current was measured from pairs of cultured bag cell neurons (Fig. 4A, inset) before and after the addition of DMSO (the vehicle) or Ca2+-liberating drugs. Introduction of either DMSO (n = 5) or 20 μM CPA (n = 6), an agent that depletes endoplasmic reticulum Ca2+ by inhibiting the Ca2+-ATPase (Seidler et al. 1989), produced no substantial change in junctional current compared with control (Fig. 4A, left and right). However, FCCP, a protonophore that collapses the mitochondrial membrane potential and causes Ca2+ to exit (Heytler and Prichard 1962), inhibited junctional current in a concentration-dependent fashion (Fig. 4B) when applied at 100 nM (n = 4), 1 μM (n = 6), or 3 μM (n = 5). The reduction was significantly greater when the two higher concentrations were compared to DMSO (Fig. 4C). Our prior work showed that the Ca2+ liberated by FCCP activated a cation current, which required ~5 min to recover (Hickey et al. 2010); hence we measured junctional current 10 min after FCCP.

To more rigorously test the role of endoplasmic reticulum Ca2+, coupled pairs were subjected to the train-stimulus after a 20- to 30-min treatment with 10 μM ryanodine or DMSO. Micromolar levels of ryanodine, an antagonist of the endoplasmic reticulum Ca2+-activated Ca2+ channel (Meissner 1985), have been shown by our laboratory to prevent Ca2+-induced Ca2+ release from cultured bag cell neurons (Geiger and Magoski 2008). Nevertheless, blocking any potential Ca2+ release during stimulation did not impact the ability of Ca2+ influx to inhibit junctional current, with DMSO and ryanodine presenting no significant difference in the level of uncoupling brought about by stimulation (Fig. 4D).

Sustained inhibition of electrical synapses depends on intracellular Ca2+. To confirm whether the prolonged decrease in coupling after the train-stimulus was due to changes in intracellular Ca2+, the Ca2+ buffering capacity of the pipette solution was enhanced by replacing the
standard intracellular saline containing 5 mM EGTA to one using either 20 mM or 40 mM EGTA (Naraghi 1997) (see MATERIALS AND METHODS for details). Neurons were dialyzed at −60 mV for 30 min, and the junctional current was studied before and after Ca\(^{2+}\) influx (Fig. 5A, inset). In control, where standard intracellular saline was in the pipettes (n = 10), the train-stimulus caused an ∼15% decrease in junctional current that lasted for at least 15 min (Fig. 5A). Subsequent to dialysis with 40 mM EGTA intracellular saline, the same stimulus failed to reduce junctional current and yielded an ∼5% increase after 15 min (Fig. 5B) (n = 6); the difference between 5 mM and 40 mM EGTA was significant (Fig. 5C). Using 20 mM EGTA-containing intracellular saline (n = 5) resulted in an ∼5% reduction in
junctional current to the train-stimulus; however, this was not significantly different from control (Fig. 5C).

CaM-kinase appears to be involved in Ca$^{2+}$-induced uncoupling. A previous description of bag cell neuron gap junctions suggested a biochemical intermediate. To determine whether CaM-kinase participates in the regulation of bag cell neuron electrical synapses, we initially employed KN-62, an isoquinolinesulfonamide-derived CaM-kinase II antagonist that is effective in *Aplysia* (Hung and Magoski 2007; Nakashashi et al. 1997; Tokumitsu et al. 1990). Pairs were prepared for 15 min prior to recording in DMSO or KN-62 and voltage-clamped for 30 min, and then junctional current was elicited (Fig. 6A, *inset*). As expected, the train-stimulus in DMSO (vehicle)-treated neurons ($n=9$) produced a $\sim-20\%$ decrease in junctional current (Fig. 6A). A similar inhibition of junctional current was observed when neurons were stimulated in 10 $\mu$M KN-62 ($n=5$; Fig. 6D); however, 50 $\mu$M KN-62 ($n=6$) prevented the uncoupling effect of Ca$^{2+}$ influx (Fig. 6B), which was statistically different from control (Fig. 6D). As further confirmation, KN-93, a methoxybenzensulfonamide that also inhibits CaM-kinase (Sumi et al. 1991), along with its structurally related but inactive analog, KN-92, were used. A 15-min incubation with 10 $\mu$M KN-93 ($n=5$) precluded the ability of Ca$^{2+}$ to lower coupling, with only an $\sim4\%$ drop (Fig. 6C). This was not the case for 10 $\mu$M KN-92, which failed to impede suppression of the electrical synapse by stimulation; the difference in the Ca$^{2+}$-induced decrease in junctional current between KN-92 and KN-93 was significant (Fig. 6E).

A PKC activator reduces junctional current. Shortly after the onset of the afterdischarge, PKC activity was shown to be upregulated and stayed elevated throughout (Conn et al. 1989a; Wayne et al. 1999). Because PKC has been established as a modulator of electrical coupling in various cells (Lampé et al. 2000; Saez et al. 1990), the effect of the phorbol ester PMA, a potent and selective PKC activator in bag cell neurons (Castagna et al. 1982; Sossin et al. 1993), was tested on junctional current (Fig. 7A, *inset*). Given that Ca$^{2+}$ influx and PKC upregulation would occur at similar times in the intact cluster (Fisher et al. 1994; Wayne et al. 1999), we also subjected these preparations to the train-stimulus. In controls, the addition of DMSO (the vehicle) to pairs of coupled neurons ($n=4$) did not appreciably alter the junctional current (Fig. 7A, *center* compared with the initial response (Fig. 7A, *left*). Subsequent delivery of the train-stimulus to these cells resulted in an $\sim-25\%$ inhibition (Fig. 7A, *right*). In contrast, introducing 100 $nM$ PMA to electrically coupled neurons ($n=13$) decreased the junctional current by $\sim15\%$ (Fig. 7B, *center* vs. *left*). Moreover, the combination of PMA and the train-stimulus was more effective at lowering electrical coupling than PMA alone and caused an $\sim35\%$ decrease in junctional current (Fig. 7B, *right*). Summary data showed a significant difference in junctional current between control pairs and those treated with PMA (Fig. 7C). Also, the PMA-induced uncoupling was additive with Ca$^{2+}$ influx, where stimulation plus PKC activation presented a significantly greater inhibition than PKC activation alone or stimulation alone (Fig. 7C).

Elevated intracellular Ca$^{2+}$ decreases amplitude and time course of electrotonic transmission. Electrical coupling mediates synchronous action potential firing of bag cell neurons throughout the afterdischarge (Blankenship and Haskins 1979; Brown et al. 1989). We sought to test the impact of Ca$^{2+}$ influx on the ETP evoked by action potentials in the presynaptic neuron. Action potential-like waveforms were generated in one neuron under voltage clamp, and the ETP was recorded in the second cell under current clamp. Idealized action potentials, based on spikes recorded during prior work from our lab, were favored over actual spikes, to avoid changes to the waveform that can occur after repetitive stimulation and Ca$^{2+}$ influx (Acosta-Urquidi and Dudek 1981; Hung and Magoski 2007). In control pairs ($n=5$), a series of voltage ramps from $-60\,mV$, mimicking the action potential, was delivered to the presynaptic neuron under voltage clamp (Fig. 8A, *bottom*), which in turn elicited a postsynaptic ETP in the coupled partner neuron under voltage clamp (Fig. 8A, *top*). Action potential-like waveforms were generated in one

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**Fig. 6.** Inhibition of calmodulin (CaM)-kinase prevents the Ca$^{2+}$-dependent decrease in electrical coupling. A: in a pair treated with DMSO and then dialyzed under voltage clamp at $-60\,mV$ for 30 min, elevating intracellular Ca$^{2+}$ with the train-stimulus to both neurons (*inset*) causes a decrease in junctional current (I2) for up to 15 min. *Inset* provides the voltage protocol to elicit junctional current. Scale bars apply to both traces. B and C: a 15-min exposure to 50 $\mu$M of KN-62, a CaM-kinase antagonist, averts the uncoupling effect of elevated intracellular Ca$^{2+}$, and, similarly, 20 min of 10 $\mu$M KN-93, a different blocker of CaM-kinase, also renders Ca$^{2+}$ influx incapable of attenuating junctional current. Scale bars apply to both traces in B and C, respectively. D: % change in junctional current after stimulation in KN-62 vs. DMSO. A 50 $\mu$M, but not 10 $\mu$M, concentration of KN-62 significantly inhibits the uncoupling effect of Ca$^{2+}$ ($F_{2,12}=7.87, P<0.01$, ANOVA; Dunnett multiple-comparisons test). E: summary data for the effect of KN-93 and its inactive analog, KN-92, on Ca$^{2+}$ entry-induced % change in junctional current. While 10 $\mu$M KN-92 does not impact uncoupling, the presence of 10 $\mu$M KN-93 eliminates any Ca$^{2+}$-evoked alteration. The change in KN-93 is significantly less than in KN-92 (unpaired Student’s t-test).
current-clamped to −60 mV with bias current (Fig. 8A, top). The control ETP lasted three to four times longer than the presynaptic waveform and did not change over time (Fig. 8A, top). However, when both neurons were given the train-stimulus \( (n = 5) \) under voltage clamp to increase \( \text{Ca}^{2+} \), the same action potential-like waveform (Fig. 8B, bottom) produced a smaller and faster ETP compared with the initial ETP (Fig. 8A, top). Both the area of the ETP as well as the time required to recover from the peak response to steady-state showed a significant decrease in stimulated vs. unstimulated control (Fig. 8, C and D). Additionally, for the stimulated pairs the input resistance was measured in the postsynaptic cell both before and after the train-stimulus, by delivering a −250 pA, 1,500-ms hyperpolarization current step from −60 mV. Postsynaptic input resistance did not change appreciably after \( \text{Ca}^{2+} \) entry (2.2 ± 15.6%).

**DISCUSSION**

Cytosolic \( \text{Ca}^{2+} \) is vital to the regulation of fundamental processes such as channel gating, excitability, and secretion (Hille 2001). Elevated intracellular \( \text{Ca}^{2+} \) decreases electrical
coupling in many excitable and nonexcitable cells (Peracchia 2004; Rohr 2004; Saez et al. 1989; Sohl and Willecke 2004; Spray and Bennett 1985). For neurons, voltage-gated Ca\textsuperscript{2+} channels are the primary means to increase Ca\textsuperscript{2+} (Catterall and Few 2008). In bag cell neurons, delivery of the 5-Hz, 1-min then 1-Hz, 5-min stimulus mimics the fast and early slow phases of the afterdischarge, leading to an immediate and prominent Ca\textsuperscript{2+} influx. The Ca\textsuperscript{2+} rise inhibits electrical transmission between pairs of cultured bag cell neurons. During the afterdischarge in the intact cluster, the fast phase sees Ca\textsuperscript{2+} change from a resting level of ~300 nM to a peak of ~800 nM, followed by a slow-phase plateau of ~450 nM (Fisher et al. 1994). For the present study, the alterations in cultured bag cell neuron intracellular Ca\textsuperscript{2+}, as monitored with fura, would approximate those seen in situ. Thus it would be reasonable to suggest that increasing Ca\textsuperscript{2+} by hundreds of nanomolar would be sufficient to close gap junctions.

Stimulation in Ca\textsuperscript{2+}-free medium is ineffective at inhibiting the electrical synapse, implicating Ca\textsuperscript{2+} entry as the cause of uncoupling. This is consistent with Chanson et al. (1999), who showed that a Ca\textsuperscript{2+}-induced reduction in junctional current between acinar cells is occluded by removing external Ca\textsuperscript{2+}. Furthermore, high-EGTA intracellular saline prevents bag cell neuron uncoupling, supporting the role of intracellular Ca\textsuperscript{2+} in electrical synapse suppression. The Ca\textsuperscript{2+}-mediated decrease in junctional conductance between hepatoma cells is also attenuated by EGTA (Lazrak and Peracchia 1993) and another Ca\textsuperscript{2+} buffer, BAPTA (Lazrak et al. 1994). There are contradictory findings as to whether Ca\textsuperscript{2+} rising in only one cell will curb electrical transmission: In coupled rat hepatocytes, imaging shows that injection of Ca\textsuperscript{2+} in one cell increases Ca\textsuperscript{2+} in the adjacent cell (Saez et al. 1989). Conversely, Rose and Loewenstein (1976) found that Ca\textsuperscript{2+} injected into a single Chironomus salivary gland cell not only fails to cause uncoupling but also does not diffuse to a neighboring cell. This parallels our finding that a train-stimulus to both bag cell neurons, rather than only one, is needed to chronically inhibit junctional current. In those pairs where we stimulated just one neuron, it is likely that Ca\textsuperscript{2+} reaches the junctional region but induces only partial uncoupling, perhaps because of a failure of Ca\textsuperscript{2+} to pass through the junction, because the Ca\textsuperscript{2+} does not fully activate the necessary transduction pathway(s), or because full closure of the gap junction requires both participating innexons to enter a shut state.

In bag cell neurons, intracellular stores contribute to Ca\textsuperscript{2+} dynamics, particularly during the slow phase of the afterdischarge (Fisher et al. 1994; Geiger and Magoski 2008). On entry, Ca\textsuperscript{2+} is taken up by the mitochondria through the Ca\textsuperscript{2+} uniporter and then released though cation exchangers to stimulate ryanodine receptors on the endoplasmic reticulum and release further Ca\textsuperscript{2+} (Geiger and Magoski 2008). When we eliminate the ability of mitochondria to clear intracellular Ca\textsuperscript{2+} with the protonophore FCCP, it causes a substantial and concentration-dependent decrease in the bag cell neuron junctional current. However, endoplasmic reticulum Ca\textsuperscript{2+} depletion by the pump inhibitor CPA does not impact coupling, nor does a high concentration of ryanodine alter the reduction in the electrical synapse brought about by stimulation. This is consistent with our prior work showing that mitochondrial, but not endoplasmic reticulum, Ca\textsuperscript{2+} has preferential access to both gate a plasma membrane cation channel and evoke neuropeptide secretion (Hickey et al. 2010, 2013). Although FCCP impacts the mitochondria, it is most probable that the drop in coupling is from Ca\textsuperscript{2+} rather than a change in cell health. This is based on the FCCP-induced current we reported previously not being due to Ca\textsuperscript{2+}-activated proteases, reactive oxygen species, or a general disruption of mitochondrial function (Hickey et al. 2010). In Chironomus salivary gland cyanide, which prevents oxidative metabolism and frees mitochondrial Ca\textsuperscript{2+} (Carafoli 1967), suppresses gap junctions (Rose and Loewenstein 1975). Conversely, blocking the endoplasmic reticulum Ca\textsuperscript{2+} pump with thapsigargin (Thastrup et al. 1990) reduces coupling between pancreatic acinar cells (Chanson et al. 1999).

Others have suggested that Ca\textsuperscript{2+}-dependent reductions in coupling are a secondary effect of Ca\textsuperscript{2+} acidifying the cytoplasm or Ca\textsuperscript{2+} synergizing with acidosis (Lazrak and Peracchia 1993). For example, Ca\textsuperscript{2+} or H\textsuperscript{+} alone does not block coupling between rat myocardial cells, but coupling decreases when acidosis accompanies Ca\textsuperscript{2+} (Burt 1987). Also, CO\textsubscript{2}-mediated cytosolic acidification lowers electrical coupling in the presence of normal Ca\textsuperscript{2+} whereas removing extracellular Ca\textsuperscript{2+} prevents this effect (White et al. 1990). Conversely, a drop in junctional communication between crayfish axons is related to a Ca\textsuperscript{2+} increase rather than low pH (Peracchia 1990). For bag cell neurons, there is evidence to rule out H\textsuperscript{+} as a factor in Ca\textsuperscript{2+}-induced uncoupling: First, substantially elevating Ca\textsuperscript{2+} in these cells by pharmacological activation of a store-operated influx pathway does not change intracellular pH (Knox et al. 2004). Second, in the present study, we included HEPES in the intracellular saline, which would work to halt any Ca\textsuperscript{2+}-evoked change in pH. Third, our intracellular dialysis with the Ca\textsuperscript{2+} chelator EGTA eliminates the effect of Ca\textsuperscript{2+}. Fourth, we show that CaM-kinase blockers prevent uncoupling.

Ca\textsuperscript{2+} might not always impact gap junctions directly but rather via a cytoplasmic intermediate. CaM is a ubiquitous Ca\textsuperscript{2+}-binding protein that can bind gap junctions (Van Eldik et al. 1985) and is well-established as an ion channel regulator (Levitan 1999). Typically, CaM inhibits gap junctions, as first suggested in Xenopus oocytes (Peracchia et al. 1983) and crayfish axons (Peracchia 1987) as well as other vertebrate (Toyama et al. 1994) and invertebrate (Arellano et al. 1988) systems. CaM can also activate CaM-kinase, which phosphorylates a wide variety of proteins (Coultrop and Bayer 2012; Wayman et al. 2008). Interestingly, CaM-kinase has been largely found to enhance junctional conductance, such as in goldfish Mauthner neurons (Pereda et al. 1998), mouse astocytes (De Pina-Benabou et al. 2001), and neuroblasts expressing rat Cx36 (Del Corso et al. 2012). We show here that CaM-kinase may have a role in Ca\textsuperscript{2+}-evoked inhibition of the bag cell neuron electrical synapse, based on the CaM-kinase antagonists KN-62 and KN-93 both preventing the uncoupling effect of Ca\textsuperscript{2+}. DeRiemer et al. (1984, 1985) found that Aplysia CaM-kinase is homologous to that in mammals and may mediate the long-lasting effect of intracellular Ca\textsuperscript{2+} entry during the afterdischarge. CaM blockers inhibit Aplysia CaM-kinase in a biochemical assay and the ability of the bag cell neurons to fire an afterdischarge. In cultured bag cell neurons, KN-62 also attenuates a prolonged depolarization and cation channel activation evoked by Ca\textsuperscript{2+} influx (Hung and Magoski 2007).

Uncoupling by Ca\textsuperscript{2+} is associated with a decrease in junctional thickness and a reduction in gap junction pore diameter (Bernardini and Peracchia 1981; Peracchia and Duhunty)
There is also evidence for so-called slow gating of gap junctions, where Ca\textsuperscript{2+}-CaM promotes more lengthy transitions between open and closed states, as well as a greater time in residual conductance states (Peracchia 2004). These changes may be due to phosphorylation (Moreno et al. 1994), and potential phosphorylation sites for various kinases have been identified in many connexins (Cruciani and Mikalsen 2002; Giepmans 2004; Laird 2005; Solan and Lampe 2005) and some innexins (Bauer et al. 2005; Potenza et al. 2002). Phosphorylation can change the unitary conductance or open probability of gap junction channels (Moreno and Lau 2007). CaM-kinase phosphorylates Cx32 on Ser residues (Saez et al. 1990) and Cx36 specifically on Ser110 and Thr111 (Alev et al. 2008). We propose that a combination of Ca\textsuperscript{2+} and protein phosphorylation regulates bag cell neuron intercellular communication. The average change in junctional conductance following voltage-gated Ca\textsuperscript{2+} influx for all control pairs in the present study was an ~25% decrease (10.5 ± 1.8 nS before vs. 7.9 ± 0.6 nS after stimulation; n = 38, P < 0.0001, Wilcoxon matched-pairs signed-ranks test). This may serve to elevate excitability by increasing system input resistance in the intact cluster. For instance, neurons could become more responsive to the cholinergic synaptic input that drives the start of the afterdischarge or the cation and persistent Ca\textsuperscript{2+} currents that maintain the depolarization (Hung and Magoski 2007; Magoski et al. 2000; Tam et al. 2009, 2011; White and Magoski 2012).

To examine the impact of reduced coupling at the network level, we modeled 100 Hodgkin-Huxley-type neurons serially connected in a row by voltage-independent gap junctions [models modified from Hodgkin and Huxley (1952), Izhikevich (2007), and Ermentrout et al. (2004); see Model development for details]. The model also included a persistent Ca\textsuperscript{2+} current (Carlin et al. 2000) to make the neurons fire tonically in a manner qualitatively similar to the bag cell neurons. Brown and Mayeri (1989) envisaged action potentials spatially spreading from neurons driven to fire in the center of the cluster at the onset of afterdischarge. Hence, model cells 35–64 received excitatory, ionotropic-type cholinergic synaptic input. The activity of all neurons was monitored for 5 s, starting with brief periodic synaptic stimulation (100 ms, 20 Hz). We initially set the junctional conductance (0.14 mS/cm\textsuperscript{2}) at just under half of the membrane conductance (0.3 mS/cm\textsuperscript{2}), as per our prior experimental findings (Dargaei et al. 2014). In this case, stimulation caused 65 of 100 neurons to spike, with excitation spreading from those receiving input to only some of the neurons down the line (Fig. 9A). However, when the junctional conductance was lowered by 25% to 0.105 mS/cm\textsuperscript{2}, mimicking Ca\textsuperscript{2+}-dependent inhibition, nearly all 100 neurons fired (Fig. 9B).
When comparing the raster plots of action potential firing in all neurons for the two conditions, the lower junctional conductance showed more gradual, albeit more effective recruitment. Furthermore, recruitment varied when junctional conductance was between 0.14 and 0.11 mS/cm², sometimes approaching all neurons while in other instances only ~70 neurons, with the performance being worse closer to 0.14 mS/cm² (Fig. 9C). This variability was due to junctional current being unable to recruit every neuron, on account of it leaking into adjacent cells. Nevertheless, when the junctional conductance was around 0.1 mS/cm², the performance of the network stabilized and all neurons consistently spiked. Not surprisingly, at low junctional conductances (<0.076 mS/cm²) there was no spatial spread of excitation and only neurons receiving synaptic input fired.

During the slow phase of the afterdischarge, PKC turns on and stays elevated (Conn et al. 1989a; Wayne et al. 1999). PKC activation enhances voltage-gated Ca²⁺ current and increases the overall amount of Ca²⁺ during the afterdischarge (DeRiemer et al. 1985). Application of the phorbol ester PMA activates Aplysia PKC, as seen in both biochemical assays of nervous system ganglia and bag cell neurons in the intact cluster or primary culture (DeRiemer et al. 1985; Sossin et al. 1993; Sossin and Schwartz 1992; Tam et al. 2011). The ability of PKC to close gap junctions has been shown in a number of cell types (Cruciani et al. 2001; Lampe et al. 2000; Spray and Bennett 1985). In our study, PMA inhibited electrical coupling, supporting the involvement of PKC in the regulation of bag cell neuron gap junctions. Inhibition was even greater when PMA-treated cells were given the train-stimulus to increase intracellular Ca²⁺. Accordingly, as Ca²⁺ falls somewhat during the slow phase, PKC could maintain inhibition by having a cumulative effect with the remaining Ca²⁺.

A reduction in coupling may also, somewhat paradoxically, promote synchrony during the afterdischarge. Ca²⁺ influx decreases the amplitude and time course of the ETP produced by a presynaptic action potential-like waveform. Typical of most gap junctions, the bag cell neuron electrical synapse is a low-pass filter (Dargaei et al. 2014), which sustains the ETP well beyond the time course of the presynaptic spike. A modest decrease in junctional conductance may improve synchrony by preventing lengthy ETPs and out-of-phase postsynaptic action potentials. The fact that postsynaptic input resistance did not change after Ca²⁺ entry suggests that the altered ETP is due to a drop in coupling. However, it is worth noting that our prior work on single cells showed an ~30% fall in input resistance (or ~45% increase in membrane conductance) after stimulation, on account of the opening of a calcium-activated, voltage-independent cation channel (Hickey et al. 2010; Hung and Magosi 2007). Thus the lack of input resistance change we observed in the present study may be due to a simultaneous loss of some junctional conductance and gain of some membrane conductance.

It is unlikely that bag cell neuron synchrony would be impared by the intermediate suppression of electrical transmission we observe here, as even weakly coupled networks synchronize (Gibson et al. 1999; Landsman et al. 2002; Traub et al. 2001). Tetanic stimulation in the thalamus also inhibits electrical synapses between reticular neurons for periods similar to what we observe in Aplysia (Landsman and Connors 2005). In acinar pancreatic cells, Ca²⁺-induced uncoupling leads to enhanced hormone secretion (Chanson et al. 1998, 1999). Furthermore, an increased ETP between electrically coupled neuroendocrine cells of cockroach is correlated to reduced juvenile hormone secretion (Lococo et al. 1986). Synchrony may be essential for ELH secretion from bag cell neurons, and a modest decrease in electrotonic transmission could help to ensure in-phase spiking and consistently timed hormone release necessary for reproduction.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: Z.D., D.S., and C.J.G. performed experiments; Z.D., D.S., C.J.G., and N.S.M. analyzed data; Z.D., D.S., and N.S.M. interpreted results of experiments; Z.D., D.S., C.J.G., and N.S.M. prepared figures; Z.D. drafted manuscript; Z.D., C.J.G., G.B., and N.S.M. approved final version of manuscript; G.B. and N.S.M. conception and design of research; G.B. and N.S.M. edited and revised manuscript.

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