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Flufenamic Acid Affects Multiple Currents and Causes Intracellular Ca^{2+} Release in *Aplysia* Bag Cell Neurons

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Gardam KE, Geiger JE, Hickey CM, Hung AY, Magoski NS. Flufenamic acid affects multiple currents and causes intracellular Ca^{2+} release in *Aplysia* bag cell neurons. *J Neurophysiol* 100: 38–49, 2008. First published April 24, 2008; doi:10.1152/jn.90265.2008. Flufenamic acid (FFA) is a nonsteroidal antiinflammatory agent, commonly used to block nonselective cation channels. We previously reported that FFA potentiated, rather than inhibited, a cation current in *Aplysia* bag cell neurons. Prompted by this paradoxical result, the present study examined the effects of FFA on membrane currents and intracellular Ca^{2+} in cultured bag cell neurons. Under whole cell voltage clamp, FFA evoked either outward (I_{out}) or inward (I_{in}) currents. I_{out} had a rapid onset, was inhibited by the K^+ channel blocker, tetraethylammonium, and was associated with both an increase in membrane conductance and a negative shift in the whole cell current reversal potential. I_{in} developed more slowly, was inhibited by the cation channel blocker, Gd^{3+}, and was concomitant with both an increased conductance and positive shift in reversal potential. FFA also enhanced the use-dependent inactivation and caused a positive-shift in the activation curve of the voltage-dependent Ca^{2+} current. Furthermore, as measured by ratiometric imaging, FFA produced a rise in intracellular Ca^{2+} that persisted in the absence of extracellular Ca^{2+} and was reduced by depleting either the endoplasmic reticulum and/or mitochondrial stores. Ca^{2+} appeared to be involved in the activation of I_{in}, as strong intracellular Ca^{2+} buffering effectively eliminated I_{in} but did not alter I_{out}. Finally, the effects of FFA were likely not due to block of cyclooxygenase given that the general cyclooxygenase inhibitor, indomethacin, failed to evoke either current. That FFA influences a number of neuronal properties needs to be taken into consideration when employing it as a cation channel antagonist.

INTRODUCTION

Flufenamic acid (FFA) was initially identified as an antiinflammatory drug by Winder et al. (1963); subsequently, Pong and Levine (1976) found it to be an inhibitor of cyclooxygenase (Cox). Beyond its effects on prostaglandin synthesis, this drug has been used more recently as a cation channel antagonist. Gogelein and Pfannmuller (1989) were the to first demonstrate that FFA inhibited nonselective cation channels, specifically in rat pancreas. Subsequently this agent has been employed as a cation channel blocker in nonneuronal preparations (Albert et al. 2006; Gogelein et al. 1990; YM Lee et al. 2003) as well as in neurons from both vertebrates and invertebrates (Bengtson et al. 2004; Cho et al. 2003; Derjean et al. 2005; Egorov et al. 2002; Ghamari-Langroudi and Bourque 2002; Green and Cottrell 1997; Haj-Dahmane and Andrade 1997; Morisset and Nagy 1999; Partridge and Valenzuela 2000; Shaw et al. 1995; Yamashita and Isa 2003).

We previously reported that FFA elicits a large outward current and potentiates a Ca^{2+}-activated cation current in the bag cell neurons of the marine mollusk, *Aplysia californica* (Hung and Magoski 2007). These neuroendocrine cells are found in two clusters at the rostral end of the abdominal ganglion, and they initiate egg-laying behavior through a long-lasting afterdischarge and neuropeptide release (Arch 1972; Dudek et al. 1979; Kupfermann 1967; Kupfermann and Kandel 1970; Loechner et al. 1990; Pinsker and Dudek 1977; Stuart et al. 1980). At least two species of nonselective, Ca^{2+}-sensitive cation channel are triggered at the onset of the afterdischarge to provide depolarizing drive for the burst. One is a voltage-dependent cation channel that is directly activated by both Ca^{2+}, through closely associated calmodulin, and phosphorylation, from closely associated protein kinase C (Lupinsky and Magoski 2006; Magoski 2004; Magoski and Kaczmarek 2005; Magoski et al. 2002; Wilson et al. 1996, 1998). The other is a voltage-independent cation channel that appears to be activated by calmodulin kinase-dependent phosphorylation (Hung and Magoski 2007). This second channel is responsible for a prolonged depolarization that can be evoked by a brief train of action potentials in cultured bag cell neurons (Hung and Magoski 2007; Whim and Kaczmarek 1998).

We have found that FFA does not block either of these well-characterized cation channels in bag cell neurons (Hung and Magoski 2007; D. A. Lupinsky and N. S. Magoski, unpublished observation). Rather it activates an outward current and actually enhances the voltage-independent cation current (Hung and Magoski 2007). In the present study, we confirm that the outward current is due to opening of a K^+ conductance. We also now show that FFA is capable of eliciting an inward current that, curiously, appears to be the result of opening a nonselective cation conductance. FFA is widely used as both a Cox inhibitor and a cation channel antagonist. Given that this drug has the potential to exert broad-spectrum effects on neuronal function, its use needs to be judicious. Alternatively, FFA may prove of value yet again as a means to activate specific conductances or cause the release of intracellular Ca^{2+} when warranted.

METHODS

Animals and cell culture

Adult *Aplysia californica* weighing 150–300 g were obtained from Marinus (Long Beach, CA) and housed in an ~300-l aquarium containing continuously circulating, aerated sea water (Instant Ocean;

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Aquarium Systems, Mentor, OH or Kent sea salt; Kent Marine, Acworth, GA) at 15°C on a 12/12 h light/dark cycle and fed Romaine lettuce five times a week.

For primary cultures of isolated bag cell neurons, animals were anesthetized by an injection of isotonic $MgCl_2$ (50% of body weight), and the abdominal ganglion was removed and treated with neutral protease (13.33 mg/ml; 165859; Roche Diagnostics, Indianapolis, IN) for 18 h at 20–22°C dissolved in tissue culture artificial sea water (tcASW; composition in mM: 460 NaCl, 10.4 KCl, 11 $CaCl_2$, 55 $MgCl_2$, 15 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 for 1 h, after which the bag cell neuron clusters were dissected from their surrounding connective tissue. Using a fire-polished Pasteur pipette and gentle trituration, neurons were dispersed in tcASW onto 35 × 10-mm polystyrene tissue culture dishes (430165; Corning, Corning, NY). Cultures were maintained in tcASW in a 14°C incubator and used for experimentation within 1–3 days. Salts were obtained from Fisher Scientific (Ottawa, ON, Canada), ICN (Aurora, OH), 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 tight-seal, whole cell method. Microelectrodes were pulled from 1.5 mm ID, borosilicate glass capillaries (TW150F-4; World Precision Instruments, Sarasota, FL) and had a resistance of 1–2 MΩ when filled with various intracellular salines. Pipette junction potentials were nulled immediately before seal formation. After seal formation, the pipette capacitive current was cancelled and, following break through, the whole cell capacitive current was also cancelled, while the series resistance (3–5 MΩ) was compensated to 80% and monitored throughout the experiment. Current was filtered at 1 kHz with the EPC-8 Bessel filter and sampled at 2 kHz using an IBM-compatible personal computer, a Digidata 1322A A/D converter (Axon Instruments/Molecular Devices, Sunnyvale, CA), and the Clampex acquisition program of pCLAMP (version 8.0; Axon Instruments/Molecular Devices). Data were gathered at room temperature (20–22°C).

Most recordings were made in normal ASW (nASW; composition as per tcASW but lacking the glucose and antibiotics) with regular intracellular saline in the pipette [composition in mM: 500 K-aspartate, 70 KCl, 1.25 $MgCl_2$, 10 HEPES, 11 glucose, 10 glutathione, 5 ethylene glycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 5 ATP (grade 2, disodium salt; A3377; Sigma-Aldrich), and 0.1 GTP (type 3, disodium salt; G8877; Sigma-Aldrich) pH 7.3 with KOH]. The free Ca^{2+} concentration of this saline was set at 300 nM by adding an appropriate amount of $CaCl_2$, as calculated by WebMaxC (http://www.stanford.edu/~cpatton/webmaxcS.htm). For experiments where intracellular Ca^{2+} was strongly buffered, the regular intracellular saline contained 20 mM EGTA and no added Ca^{2+}. A junction potential of 15 mV was calculated for these intracellular salines versus nASW and compensated for by subtraction off-line.

Ca^{2+} currents were isolated using an ASW where Na^+ was replaced with tetraethylammonium (TEA) and K^+ with Cs^+ (composition in mM: 460 TEA-Cl, 10.4 CsCl, 55 $MgCl_2$, 11 $CaCl_2$, 15 HEPES, pH 7.8 with CsOH). The protocol also employed an intracellular saline where the K^+ was replaced with Cs^+ (composition in mM): 70 CsCl, 10 HEPES, 11 glucose, 10 glutathione, 5 EGTA, 500 aspartic acid, 5 ATP, and 0.1 GTP, pH 7.3 with CsOH. In some instances, on-line leak subtraction was performed using a P/4 protocol from −60 mV with subpulses of opposite polarity and one-fourth the magnitude, an inter-subpulse interval of 500 ms, and 100 ms before actual test pulses. In other cases, 10 mM Ni^{2+} ($NiCl_2$; N6136; Sigma-Aldrich) was used to completely block the Ca^{2+} current (Hung and Magoski 2007), and this remaining, Ni^{2+}-insensitive current was subtracted from the prior current to remove leak. A junction potential of 20 mV was compensated for by subtraction off-line.

Intracellular Ca^{2+} measurements

Somatic intracellular Ca^{2+} was measured by ratiometric imaging of the dye, fura PE3 (K^+ salt; 0110; Teflabs, Austin, TX) (Vorndran et al. 1995). Fura-PE3 was pressure injected via sharp electrodes using a PMI-100 pressure microinjector (Dagan, Minneapolis, MN), while simultaneously monitoring membrane potential with an Axoclamp 2B amplifier (Axon Instruments/Molecular Devices). Microelectrodes were pulled from 1.2 mm ID, borosilicate glass capillaries (1B120F-4; World Precision Instruments) and had a resistance of 30–50 MΩ when the tip was filled with 10 mM fura-PE3 then backfilled with 3 M KCl. Injections usually required 10–15 300- to 900-ms pulses at 30–60 kPa to fill the neurons with an optimal amount of dye (estimated at 50–100 μM). All neurons used for imaging showed resting potentials of −50 to −60 mV and displayed action potentials that overshot 0 mV following depolarizing current injection (0.5–1 nA, directly from the amplifier). After dye injection, neurons were allowed to equilibrate for ≥30 min.

Ca^{2+} imaging was performed using a Nikon TS100-F inverted microscope (Nikon, Mississauga, ON, Canada) equipped with a Nikon Plan Fluor ×10 objective (NA = 0.3). The light source was a 75 W Xenon arc lamp and a multi-wavelength DeltaRAM V monochromatic illuminator (Photon Technology International, London, ON, Canada) coupled to the microscope with a UV-grade liquid-light guide. Between acquisition episodes, the excitation illumination was blocked by a shutter, which along with the excitation wavelength was controlled by a IBM-compatible personal computer, a Photon Technology International computer interface, and ImageMaster Pro software (version 1.49; Photon Technology International). The emitted light passed through a 510/40-nm barrier filter prior to being detected by a Photon Technology International IC200 intensified charge coupled device camera. The camera intensifier voltage was set based on the initial fluorescence intensity of the cells at the beginning of each experiment and maintained constant thereafter. The camera black level was set prior to an experiment using the camera controller such that at a gain of 1 there was a 50:50 distribution of blue and black pixels on the image display with no light going to the camera. The ratioed image of the fluorescence intensities (converted to pixel values) from 340 and 380 nm excitation wavelengths was derived and averaged four to eight frames per acquisition, resulting in a single full-frame (520 × 480 pixels) acquisition time of 0.5–4 s. A sample of the fluorescence intensities ratio was taken typically at 1-min intervals using regions of interest (ROIs) defined over the neuronal somata prior to the start of the experiment. The ratio was recorded as 340/380 to reflect free intracellular Ca^{2+}. The black level determination, image acquisition, frame averaging, emitted light ROI sampling, and ratio calculations were carried out using the ImageMaster Pro software. Ratio calculations were saved for subsequent analysis (see following text). Imaging was carried out at room temperature (20–22°C) and performed in both nASW and Ca^{2+}-free ASW (cfASW; composition as per nASW but with $CaCl_2$ omitted and 0.5 mM EGTA added).

Drug application and reagents

The culture dish served as the bath with salines and/or drugs being applied using a gravity-driven perfusion system of ~1 ml/min. In some cases, drugs were introduced directly into the bath by pipetting a small volume (<10 μl) of concentrated stock solution or a larger volume of saline (~500 μl) that was initially removed from the bath, mixed with the stock solution, and then reintroduced. Care was taken to perform all pipetting near the side of the dish and as far away as possible from the neuron(s). $GdCl_3$ (G-7532; Sigma-Aldrich) and tetraethylammonium-Cl (TEA; AC150905000; Fisher) were dissolved directly into nASW. *N*-(3-[trifluoromethyl]phenyl)anthranilic flufenamic acid (FFA; F9005; Sigma-Aldrich) was dissolved in ethanol, while dimethyl sulfoxide (DMSO; BP231-1; Fisher) was used to dissolve bafilomycin A (B1793; Sigma-Aldrich), cyclopiazonic acid

(CPA) (C1530; Sigma-Aldrich or 239805; Calbiochem; San Diego, CA), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; C2920; Sigma-Aldrich), indomethacin (I7378; Sigma-Aldrich), and paxilline (P-2928; Sigma-Adrich). The maximal final concentration of DMSO or ethanol was 0.01 μM or 0.01%, respectively, which in control experiments had no effect on intracellular Ca^{2+} or membrane properties.

Analysis

The Clampfit analysis program of pCLAMP (Axon Instruments/Molecular Devices) was used to determine the amplitude and time course of currents evoked by FFA. Cursors were placed at the baseline current, prior to FFA delivery, as well as at the peak after the drug. The difference between the two cursor values was taken as the peak amplitude. Conductance was derived using Ohm's law ($G = I/V$) from the current during a 200-ms step from −60 to −70 mV. The percentage change was calculated from the conductance before and after FFA delivery. The current-voltage relationship of the Ca^{2+} current was determined by measuring peak current between cursors set at the start and end of the traces in Clampfit. Current was normalized to cell size by dividing by the whole cell capacitance (as determined by the EPC-8 slow capacitance compensation circuitry) and plotted against voltage using Origin (version 7.0; OriginLab, Northampton, MA). Activation curves were made by dividing the Ca^{2+} current amplitude at all voltages by that at +10 mV (the peak current voltage). These curves were fit with Boltzmann functions using Origin to derive the half-maximal voltage of activation ($V_{1/2}$; the voltage required to recruit half of the maximum current), and the slope factor (k; the amount of voltage required to shift the $V_{1/2}$ e-fold). For intracellular Ca^{2+}, Origin was used to import and plot ImageMaster Pro files as line graphs. Values were derived from changes determined by eye or with adjacent-averaging from regions that had reached steady-state for 3–5 min.

Data are presented as the means ± SE as calculated using either Origin or Instat (version 3.05; GraphPad Software; San Diego, CA). Statistical analysis was performed using Instat. The Kolmogorov-Smirnov method was used to test data sets for normality. A one-sample t-test was used to determine if the mean of a single group was different from zero. Paired and unpaired Student's t-test (standard or Welch corrected) or the Mann-Whitney test was used to test whether the mean differed between two groups. Comparisons between three or more means used a standard one-way ANOVA with Dunnett's multiple comparisons post hoc test. All tests were two-tailed. Data were considered significantly different if the P value was <0.05.

RESULTS

FFA activates a large outward current in Aplysia bag cell neurons

Initially we set out to confirm and characterize the outward current activated by FFA as previously reported by Hung and Magoski (2007). Cultured bag cell neurons were whole cell voltage-clamped using regular intracellular saline in the pipette (K^+-aspartate based with 300 nM free Ca^{2+}) and nASW in the bath. At a holding potential of −60 mV, application of 300 μM FFA elicited a prominent outward current (I_{out}) that was, on average, 1.75 nA with a time to peak of <1 min ($n = 8$; Fig. 1, *A* and *B*). Given that many outward currents pass K^+, the effects of a common K^+ channel blocker, TEA (Hagiwara and Saito 1959), was examined on I_{out}. After allowing I_{out} to fully develop, 50 mM TEA was perfused along with the FFA. This consistently resulted in the current completely returning to baseline - typically near zero ($n = 6$; Fig. 1*C*). When Hung and

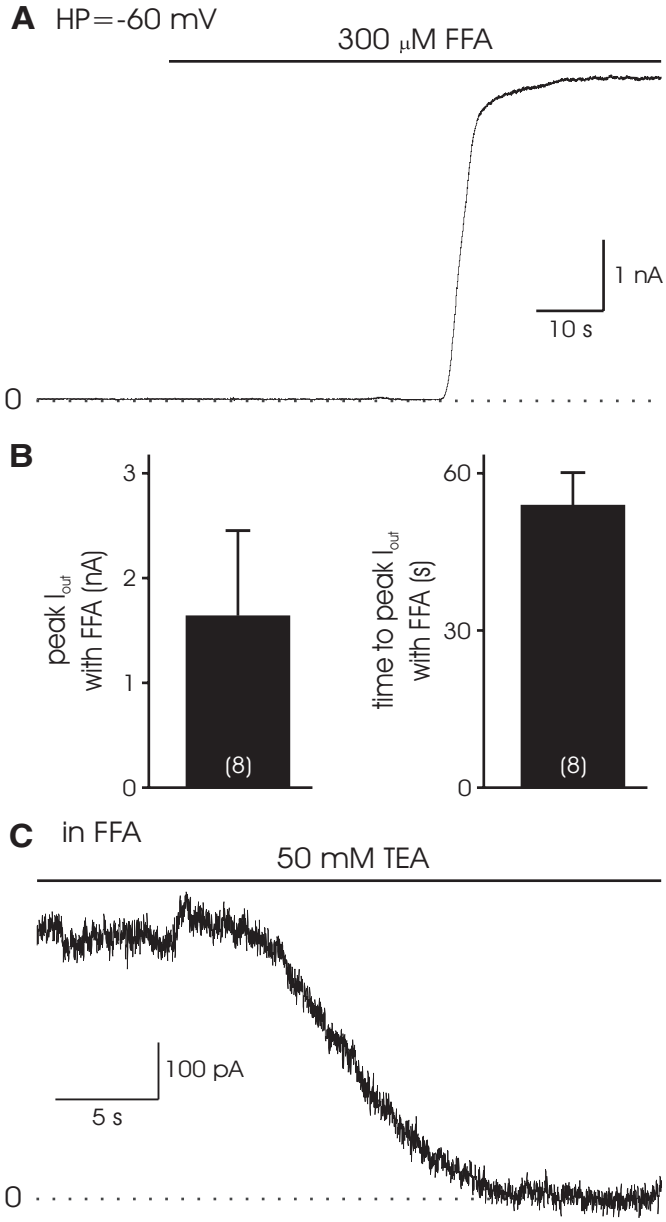

FIG. 1. Flufenamic acid (FFA) activates an outward current that is sensitive to TEA. *A*: perfusion of 300 μM FFA onto a cultured bag cell neuron, voltage-clamped at −60 mV, elicits a prominent outward current. *B, left*: summary amplitude data showing that the average peak outward current (I_{out}) is ~1.5 nA. *Right*: summary time course data indicating that I_{out} develops relatively quickly and reaches peak amplitude within 1 min. *C*: after allowing I_{out} to fully activate in the presence of 300 μM FFA, simultaneous perfusion of 50 mM TEA results in a return to baseline for the current (representative of $n = 6$).

Magoski (2007) first described the effects of FFA on bag cell neurons, they showed that concentrations between 100 and 200 μM also activated I_{out}, although the current was larger and more reliably evoked with 300 μM. Moreover, concentrations >300 μM had a negative impact on neuronal viability, perhaps due to the effects of FFA on intracellular Ca^{2+} (see following text). As such, we have used a concentration of 300 μM throughout the present study. When used as a cation channel blocker, for both vertebrate and invertebrate cells, FFA is typically employed at 100–500 μM (Derjean et al. 2005; Ghamari-Langroudi and Bourque 2002; Green and Cottrell

1997; Morisset and Nagy 1999; Partridge and Valenzuela 2000; Shaw et al.1995).

The susceptibility of I_{out} to TEA suggested that it was mediated by the opening of a K^+ conductance. To test this, the reversal potential and membrane conductance were determined under control conditions and once peak I_{out} had been reached in the presence of FFA. Specifically, both a 200-ms step from −60 to −70 mV (see Fig. 2A, *bottom*) and a 10-s ramp from −120 to 0 mV (see Fig. 2*B, inset*) were delivered. Changes in membrane conductance were calculated from the current during the step, while whole cell current reversal potential was derived from point where the current crossed the abscissa. During the FFA-evoked I_{out}, the whole cell conductance increased almost 40-fold, consistent with ion channel opening ($n = 8$; Fig. 2, *A* and *C, right*). The current-voltage relationship in the presence of FFA was primarily outward in nature and showed only weak voltage dependence with outward rectification at potentials more positive than −40 mV ($n = 7$; Fig. 2*B*). Compared with control, the reversal potential of the whole cell current was shifted in the negative direction (from approximately −65 mV to nearly −80 mV; Fig. 2, *B* and *C, left*) to an extent that reached statistical significance.

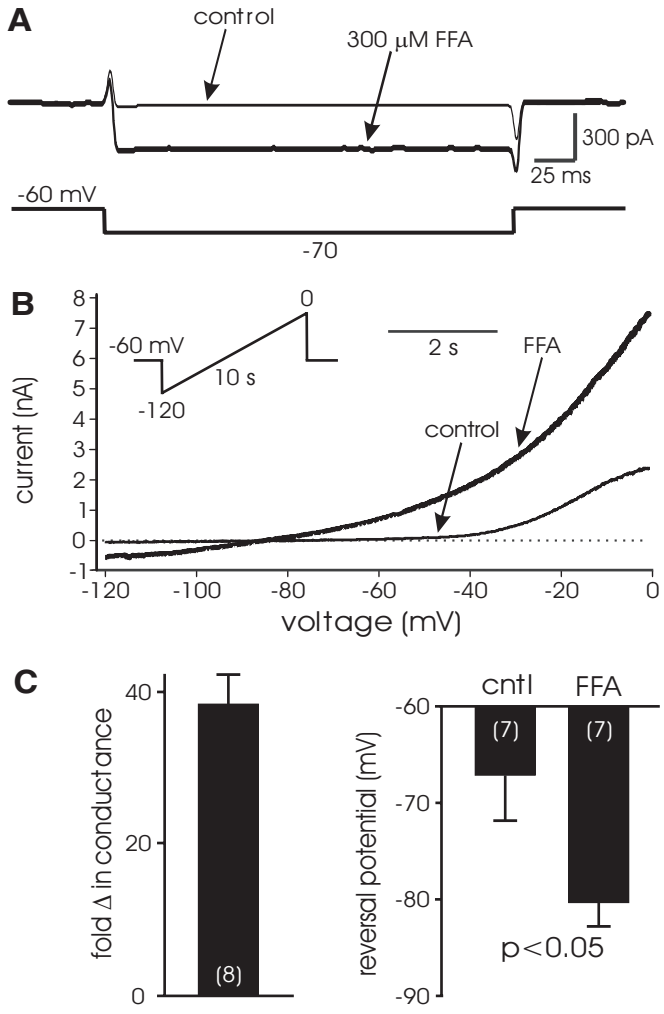

FIG. 2. The outward current activated by FFA is consistent with opening of a K^+ channel. *A*: the whole cell conductance rises markedly following addition of 300 μM FFA. Sample traces, taken during control and at the peak of I_{out}, show that FFA increases the current flowing during a test pulse to −70 mV from a holding potential of −60 mV. Although after FFA there is a large increase in holding current, the traces have been aligned for comparison. *B*: in FFA, the whole cell current shifts to a more negative reversal potential and is dominated by an outward component. During a 10-s voltage ramp from −120 to 0 mV (see *inset*), again taken at control vs. peak I_{out}, the FFA current is prominently outward, roughly linear over much of the voltage range, and reverses near −90 mV. *C, left*: summary graph of the change in conductance shows a nearly 40-fold elevation with FFA, suggesting that channels open with the drug. *Right*: summary graph of the significant negative shift in reversal potential, toward E_K, of the current evoked by the −120 to 0 mV ramp (paired Student's *t*-test).

FFA activates a small inward current

In addition to I_{out}, approximately half of the neurons displayed a much smaller inward current (I_{in}) on exposure to 300 μM FFA during recording conditions identical to that described in the preceding text ($n = 13$; Fig. 3*A*). The two currents were not observed in the same individual neuron although a given group of neurons from a single animal could yield cells that responded to FFA with I_{out} *or* I_{in}. The mean amplitude of I_{in} was close to 300 pA and, in comparison to I_{out}, showed a slower time to peak at just under 4 min (Fig. 3*B*). Recognizing that I_{in} is small compared with I_{out}, we sought to ascertain if I_{out} was obscuring I_{in}. Using a set of neurons that consistently displayed an I_{out} under control conditions, TEA was applied before delivery of FFA. However, the application of 300 μM FFA in the presence of 50 mM TEA did not reveal a inward component ($n = 5$; data not shown).

One possible source of I_{in} is a nonselective cation channel—multiple forms of which are found in bag cell neurons (Hung and Magoski 2007; Knox et al. 1996; Wilson et al. 1996). Thus Gd^{3+}, an established cation channel blocker (Chakfe and Bourque 2000; Franco and Lansman 1990; Popp et al. 1993; Yang and Sachs 1989), was added after the FFA-evoked I_{in} had reached peak. The introduction of 100 μM Gd^{3+} in the presence of FFA resulted in clear attenuation of I_{in} (Fig. 3*B*; $n = 5$). We further explored if I_{in} was mediated by a nonselective cation conductance by examining the reversal potential and membrane conductance before and after FFA. As was performed for I_{out}, both a 200-ms step from −60 to −70 mV (see Fig. 4*A, bottom*) and a 10-s ramp from −120 to 0 mV (see Fig. 4*B, inset*) were delivered to measure conductance and reversal potential, respectively. At peak FFA-evoked I_{in}, the whole cell conductance rose more than sixfold, in agreement with ion channel opening ($n = 13$; Fig. 4, *A* and *C, right*). Furthermore, under these conditions, the current-voltage relationship was dominated by a largely inward and voltage-independent component that only showed rectification only after reversal to the outward phase ($n = 13$; Fig. 4*B*). In contrast with control conditions, the reversal potential of the whole cell current was positively shifted (from around −70 mV to just over −15 mV; Fig. 4, *B* and *C, left*) such that it reached statistical significance.

FFA alters voltage-gated Ca^{2+} current

The prior observation that FFA potentiated a Ca^{2+}-activated cation current in bag cell neurons (Hung and Magoski 2007) led us to consider that FFA could be exerting an affect on voltage-gated Ca^{2+} influx. This is to say, the cation current could have been enhanced indirectly by upregulating Ca^{2+} channels. Under conditions where cultured bag cell neuron voltage-gated Ca^{2+} current was isolated (see METHODS)

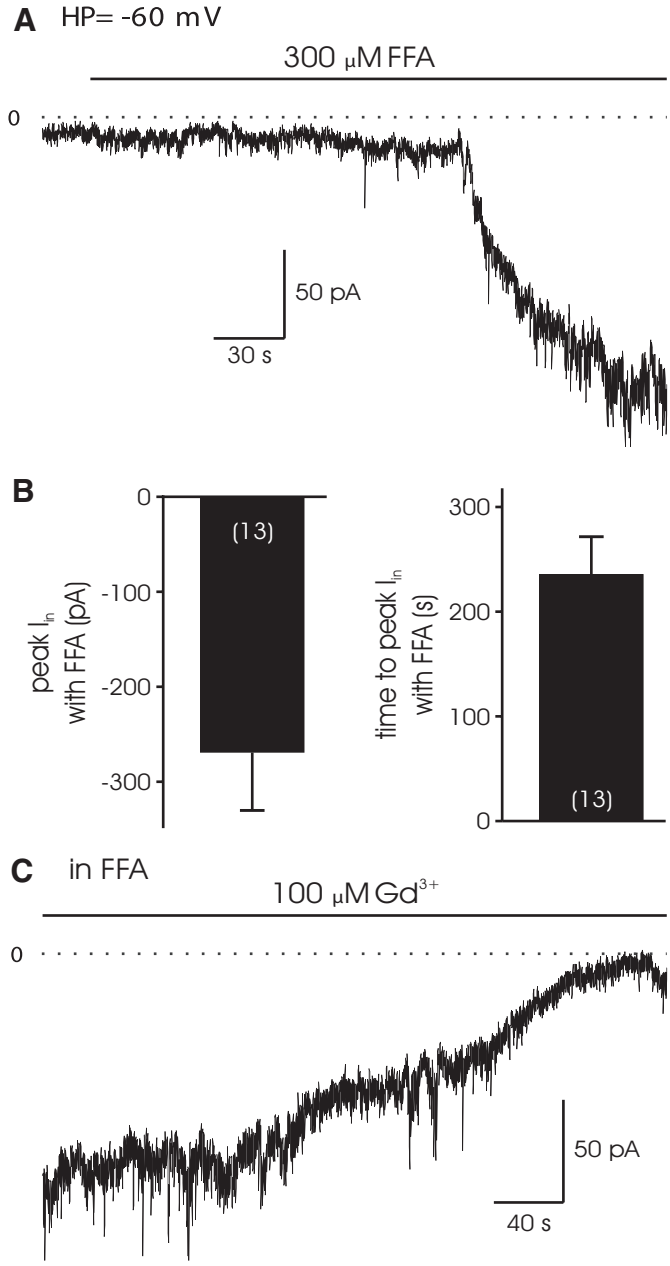

FIG. 3. FFA activates an inward current that is sensitive to Gd^{3+}. *A*: perfusion of 300 μM FFA onto a bag cell neuron, voltage-clamped at −60 mV, elicits a modest inward current. *B, left*: summary amplitude data showing that the average peak inward current (I_{in}) is nearly 300 pA. *Right*: summary time course data indicating that I_{in} develops more slowly as compared with I_{out} and reaches peak amplitude within 4 min. *C*: in the presence of 300 μM FFA, fully activated I_{in} is completely blocked by simultaneous perfusion of 100 μM Gd^{3+} (representative of $n = 5$).

(DeRiemer et al. 1985; Hung and Magoski 2007), we observed a strongly voltage-dependent Ca^{2+} current that activated between −30 and −20 mV, peaked near +10 mV, and showed moderate inactivation over 200-ms test pulses (Fig. 5*A*). Delivery of 100 μM FFA did not alter Ca^{2+} current amplitude or activation characteristics ($n = 4$; data not shown). However, compared with ethanol controls ($n = 3$), addition of 300 μM FFA ($n = 4$) markedly decreased the Ca^{2+} current during a 5-Hz train of 100-ms voltage steps from −60 to +10 mV (Fig. 5*B*). Thus over the course of the voltage train, FFA appeared to block voltage-gated Ca^{2+} channels in a use-dependent manner. This voltage train was the same as that used by Hung and Magoski (2007) to evoke the Ca^{2+}-activated cation current first observed to be potentiated by FFA. Parenthetically, the Ca^{2+} current activation curve, following the FFA-induced use-dependent block, displayed a rightward shift in half activation ($V_{1/2}$; from −8.5 to −6.3 mV) with little change in sensitivity (*k;* from 4.5 to 5.1; Fig. 5*C*).

FFA causes release of intracellular Ca^{2+}

An inhibition of voltage-gated Ca^{2+} current does not explain how FFA is able to both evoke I_{out} and I_{in}, as well potentiate

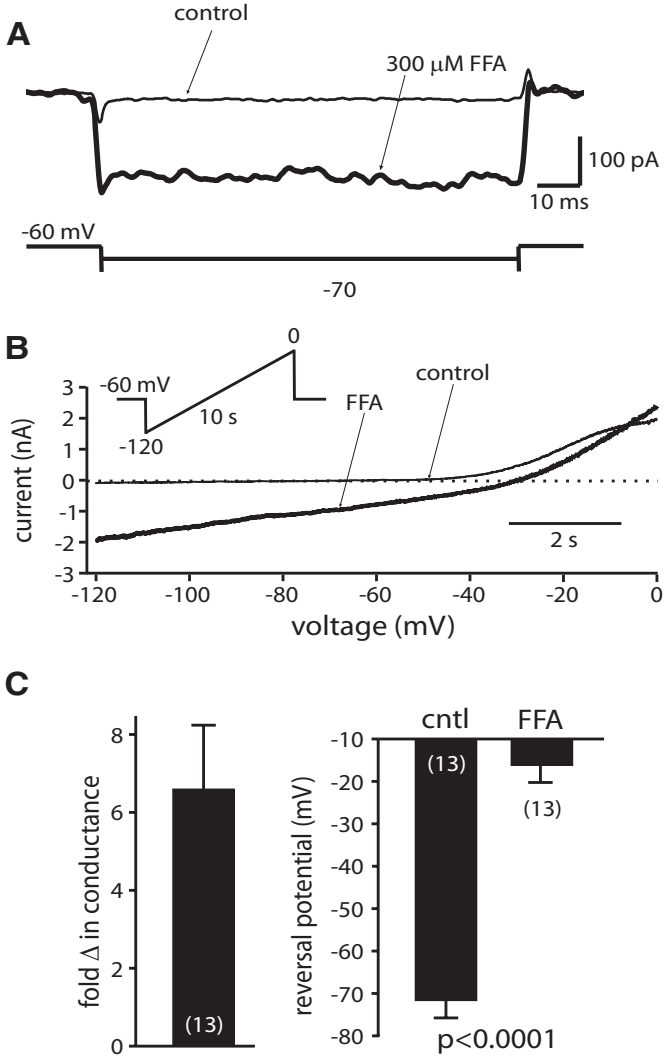

FIG. 4. The inward current activated by FFA is consistent with opening of a nonselective cation channel. *A*: there is an increase in whole cell conductance after perfusion of 300 μM FFA. Sample traces, taken during control and at the peak of I_{in}, show that FFA increases the current flowing during a test pulse to −70 mV from a holding potential of - 60 mV. Again, the traces have been aligned for comparison. *B*: in FFA, the whole cell current shifts to a more positive reversal potential and displays a very prominent inward component. During a 10-s voltage ramp from −120 to 0 mV (see *inset*), again taken at control vs. peak I_{out}, the FFA current is largely inward, essentially linear over the majority of the voltage range, and reverses near −30 mV. *C, left*: summary graph of the change in conductance shows greater than a sixfold increase with FFA, suggesting that channels open with the drug. *Right*: summary graph of the significant positive shift in reversal potential, toward 0 mV, of the current evoked by the −120 to 0 mV ramp (paired Student's *t*-test).

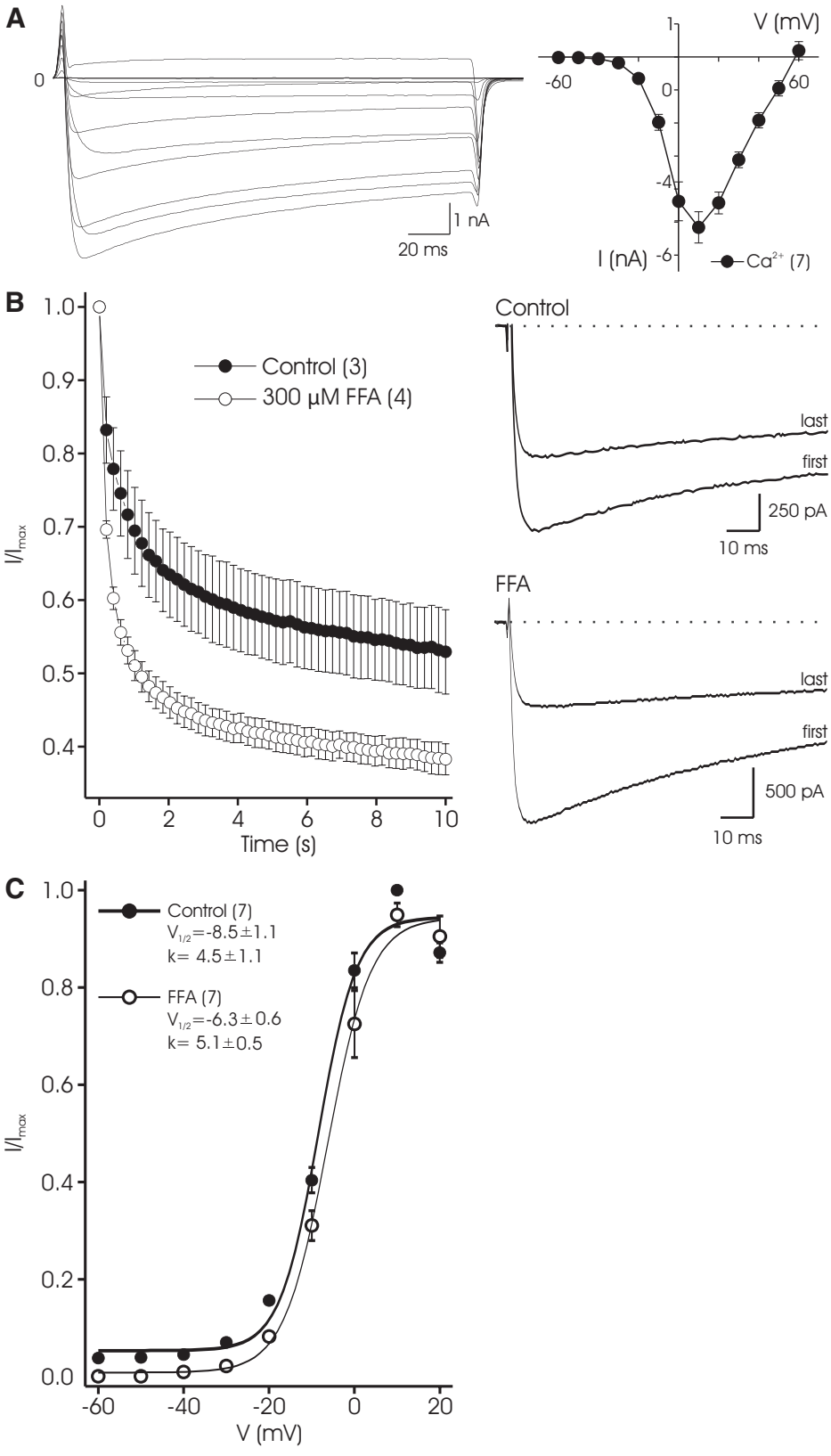

FIG. 5. The voltage-gated Ca^{2+} current is altered by FFA. *A, left*: typical whole cell, voltage-gated Ca^{2+} currents evoked by 200-ms steps from a holding potential of −60 up to +60 mV in 10-mV increments. *Right*: current-voltage relationship for 7 neurons plotting peak current vs. test potential shows that the maximum Ca^{2+} current occurs at +10 mV. *B*: use-dependent inactivation of the Ca^{2+} current is enhanced with perfusion of 300 μM FFA. *Left*: summary graph of peak current evoked during a 5-Hz, 10-s train of 100-ms test pulses to +10 mV from a holding potential of −60 mV. Compared with control, the Ca^{2+} current in FFA undergoes more rapid use-dependent inactivation, particularly early in the train. This is best illustrated by the current flowing during pulses 2–4, which in FFA show prominent, successive reductions. In both cases, data are normalized to the current during the first pulse. *Right*: sample current traces evoked by the first and last test pulse in control and the presence of FFA. The amount of reduction in the peak of the first compared with the last current is greater with FFA. *C*: activation curves for Ca^{2+} current in control and the presence of FFA. Current is normalized by dividing peak current at each test voltage by the peak current at +10 mV. Voltage protocol as per *A*. A Boltzmann function fit of the points shows very similar voltage-dependent activation properties, with FFA producing a small, positive shift in the half-activation ($V_{1/2}$) without an appreciable change in sensitivity (k).

the Ca^{2+}-activated cation current reported by Hung and Magoski (2007). Alternatively, a mechanism may be found in prior work showing that FFA can cause release of intracellular Ca^{2+} in *Helix* and hippocampal neurons as well as a mandibular cell line (Lee et al. 1996; Partridge and Valenzuela 2000; Poronnik et al. 1992; Shaw et al. 1995). We used ratiometric imaging of fura PE3-loaded cultured bag cell neurons to examine if 300 μM FFA altered intracellular Ca^{2+}. In Ca^{2+}-containing nASW, FFA produced a clear elevation of intracellular Ca^{2+} that reached a stable peak in <10 min (n = 11;

Fig. 6*A*). To determine if the Ca^{2+} increase was due to release of intracellular Ca^{2+} or influx of extracellular Ca^{2+} (possibly through I_{in} or voltage-gated Ca^{2+} current activated by depolarization), FFA was introduced in cfASW. Under those conditions, the FFA-induced elevation of intracellular Ca^{2+} was unaltered although in some instances, it was slower to reach peak amplitude ($n = 11$; Fig. 6*B*).

That removal of extracellular Ca^{2+} did not prevent the FFA-induced Ca^{2+} elevation, suggested the response was due to liberation of intracellular Ca^{2+}. To ascertain which store(s) were involved, bag cell neurons were bathed in cfASW and pretreated with agents known to deplete Ca^{2+} from specific intracellular stores. Prior application of 20 μM of either the endoplasmic reticulum Ca^{2+}-ATPase blocker, CPA (Seidler et al. 1989), or the protonophore, FCCP (Heytler and Prichard 1962) diminished the FFA-induced Ca^{2+} elevation to an extent where it reached significance in comparison to cfASW alone ($n = 16$ and 20; Fig. 6, *C, D,* and *F*). Dual depletion with both CPA and FCCP together essentially eliminated the FFA-induced Ca^{2+} elevation ($n = 7$; Fig. 6, *E* and *F*). CPA depletes Ca^{2+} from the endoplasmic reticulum (see Verkhratsky 2005 for review), including that of bag cell neurons (Kachoei et al. 2006); furthermore, FCCP collapses the mitochondrial proton gradient, resulting in the leak of Ca^{2+} out of that organelle (Collins et al. 2000; Simpson and Russell 1996), a phenomenon also observed in bag cell neurons (Jonas et al. 1997). No significant difference was observed following pretreatment with 100 nM of the vesicular H^+-ATPase inhibitor, bafilomycin

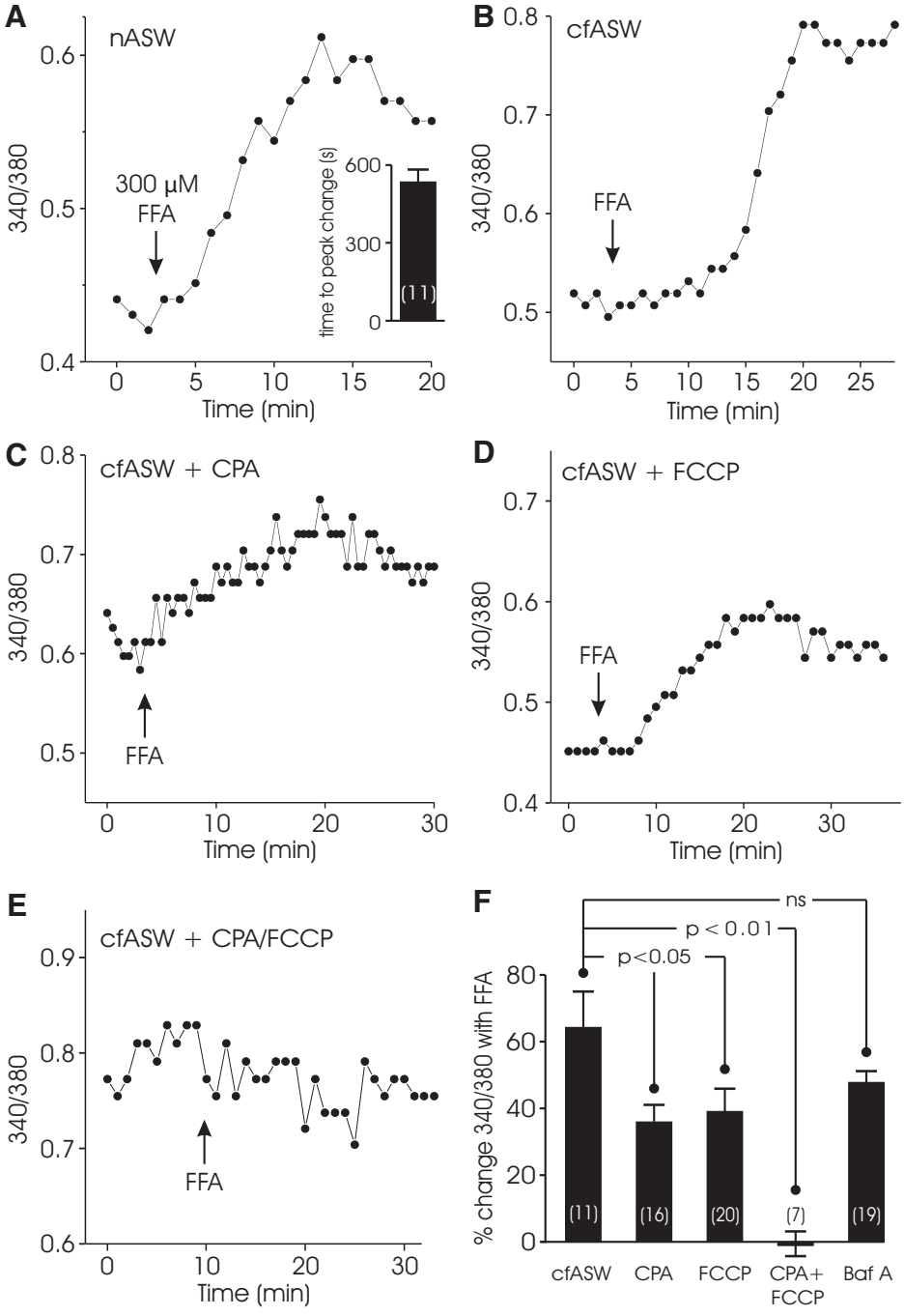

FIG. 6. FFA increases intracellular Ca^{2+}. *A*: in Ca^{2+}-containing normal artificial seawater (nASW), bath application 300 μM FFA causes a relatively rapid rise in intracellular Ca^{2+} as monitored by ratiometric imaging of fura PE3. *Inset*: summary time course data shows the FFA-induced Ca^{2+} increase reaches peak amplitude in ~8 min. *B*: when extracellular Ca^{2+} is removed and FFA applied while bathing the neurons in Ca^{2+}-free ASW (cfASW), the rise in intracellular Ca^{2+} persists. Although more variable, the time course is similar to that seen in nASW. *C*: pretreatment with 20 μM of the endoplasmic reticulum Ca^{2+}-ATPase blocker, cyclopiazonic acid (CPA), attenuates the FFA-induced Ca^{2+} increase in cfASW. *D*: similarly, prior application of 20 μM of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), an agent that collapses the mitochondrial membrane potential, reduces the FFA-induced Ca^{2+} increase in cfASW. *E*: if both the ER and the mitochondria are depleted by simultaneous application of CPA and FCCP, the ability of FFA to elevate Ca^{2+} in cfASW is largely abolished. Note that while the absolute values on the ordinate for *B–E* are not necessarily the same, the range is identical in all cases. *F*: summary graph of the FFA-induced Ca^{2+} increase in cfASW alone as well as cfASW with CPA, FCCP, CPA plus FCCP, or bafilomycin A (Baf A; 100 nM). The latter is a vesicular H^+-ATPase inhibitor that depletes Ca^{2+} from acidic stores. The response to FFA is significantly reduced by pretreatment with CPA, FCCP, or CPA plus FCCP but not Baf A (standard ANOVA followed by Dunnett's multiple comparisons test of cfASW vs. cfASW with CPA, FCCP, CPA plus FCCP, or Baf A).

A (n = 19; Fig. 6*F*). The latter is a vacuolar H^+-ATPase inhibitor (Bowman et al. 1988) that depletes Ca^{2+} from acidic stores (Christensen et al. 2002; Goncalves et al. 1999) and is established as being effective in bag cell neurons by our laboratory (Kachoei et al. 2006).

FFA-evoked inward current, but not the outward current, depends on intracellular Ca^{2+}

The release of intracellular Ca^{2+} by FFA raises the possibility that I_{out} and/or I_{in} may be gated by cytosolic Ca^{2+}. For I_{out}, we tested this by recording the FFA-induced current while dialyzing cultured bag cell neurons with either regular intracellular saline (5 mM EGTA) or a high EGTA (20 mM) intracellular saline in the whole cell pipette. The prediction being that if I_{out} was Ca^{2+} sensitive, the high EGTA would buffer the Ca^{2+} released by FFA and prevent activation. However, there was no difference between the magnitude of I_{out} recorded with the two internal salines (n = 5 and 5; Fig. 7, *A, B,* and *D*). Moreover, delivery of 10 μM paxilline, a Ca^{2+}-activated K^+ channel blocker (Knaus et al. 1994) known to be effective in bag cell neurons (Zhang et al. 2002) did not alter I_{out} (n = 5; Fig. 7, *C* and *D*). With respect to I_{in}, when FFA was used to evoked the current in a different group of neurons, it proved sensitive to high EGTA intracellular saline. While control neurons dialyzed with regular intracellular saline all displayed an I_{in} (n = 9; Fig. 7*E*), the cells recorded using high EGTA in the pipette failed to display any current change (n = 7; Fig. 7*F*; see *inset* for quantification).

Inhibition of cyclooxygenase (Cox) does not evoke either current

A final option for a mechanism that could generate I_{out} and/or I_{in} is the inhibitory effect of FFA on Cox (Pong and Levine 1976). Potentially, a decrease in the resting prostaglandin level could remove some steady-state inhibition and open I_{out} and/or I_{in}. The inhibitory action of FFA does not distinguish between Cox-1 and -2 isoforms (Ouellet and Percival 1995). As such, we employed indomethacin, a general Cox antagonist (Laneuville et al. 1994)

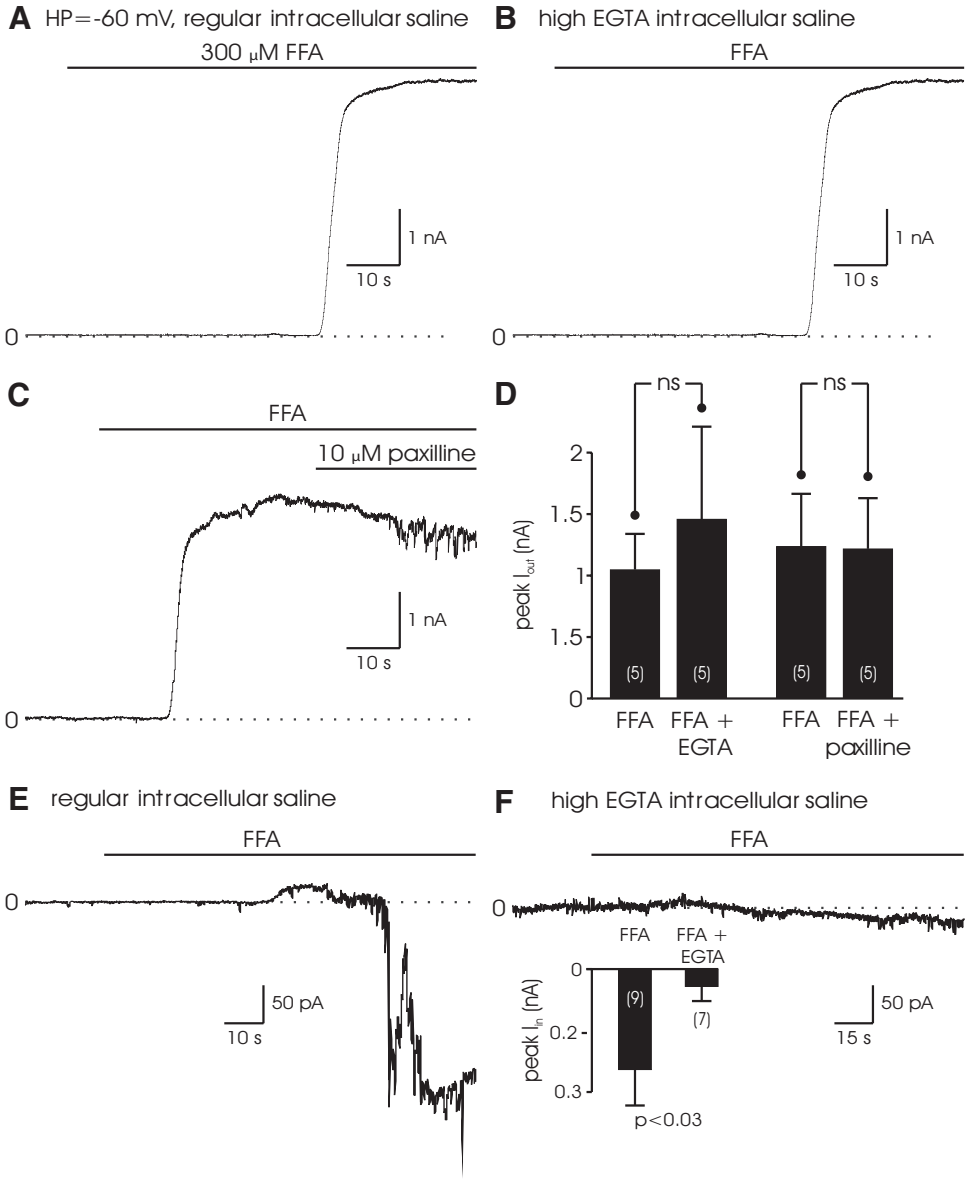

FIG. 7. I_{out} appears Ca^{2+}-independent, whereas I_{in} appears Ca^{2+}-dependent. *A*: while voltage-clamping at −60 mV and dialyzing with regular intracellular saline, perfusion of 300 μM FFA elicits a prominent I_{out}. *B*: in a parallel experiment, dialysis with intracellular saline containing high (20 mM) EGTA does not occlude I_{out}. *C*: following activation of I_{out} by FFA (regular intracellular saline dialysis), introduction of 10 μM paxilline does not alter the steady-state current. *D*: summary graph showing no significant difference between I_{out} in neurons dialyzed with regular intracellular saline and those dialyzed with high EGTA intracellular saline (unpaired Student's t-test, Welch corrected). Similarly, application of 10 μM paxilline, a Ca^{2+}-activated K^+ channel blocker, does not significantly decrease FFA-induced I_{out} (paired Student's t-test). *E*: in a different group of neurons from those used in *A–D*, FFA elicits a typical I_{in} when the cell is dialyzed with regular intracellular saline (representative of n = 9). *F*: attempting to evoke I_{in} during a parallel experiment, involving dialysis with high EGTA intracellular saline, fails to elicit any change in current (representative of n = 7). *Inset*: summary graph shows a significant difference between control and high EGTA intracellular conditions (unpaired Student's t-test, Welch corrected).

that is known to inhibit prostaglandin synthesis in *Aplysia* nervous tissue (Piomelli et al. 1987a). When 10 μM indomethacin was applied to cultured bag cell neurons voltage-clamped at −60 mV in nASW, it produced no change in the holding current ($n = 7$; Fig. 8*A*). However, following washout of the indomethacin, delivery of 300 μM FFA to those same neurons elicited either I_{out} or I_{in} ($n = 4$ and 3; Fig. 8*B*).

DISCUSSION

FFA is considered an antagonist of nonselective cation channels (Gogelein and Pfannmuller 1989), including some (Albert et al. 2006; YM Lee et al. 2003) but not all (Hill et al. 2006; Ohki et al. 2000), transient receptor potential (TRP) channels. However, appreciable evidence points to additional effects of FFA, such as inhibiting Ca^{2+}-activated Cl^- channels in oocytes (White and Aylwin 1990), voltage-gated Na^+ channels in dorsal root ganglion neurons (HM Lee et al. 2003), and connexins in cell lines and astrocytes (Harks et al. 2001; Srinivas and Spray 2003; Ye et al. 2003). FFA is also an indiscriminate inhibitor of Cox (Ouellet and Percival 1995). Yet in the present study, it is apparent that the effects of FFA manifest through a direct action on a K^+ conductance (I_{out}) and an indirect action, via intracellular Ca^{2+} release, on a cation conductance (I_{in}). Because the two conductances were never seen simultaneously in the same neuron, and blocking I_{out} did not reveal I_{in}, we believe that the two conductances, or the pathways leading to their activation, are differentially expressed. The inability of the chemically unrelated Cox inhibitor, indomethacin (Laneuville et al. 1994), to activate either current, suggests that a change in prostaglandin levels is not the underlying mechanism for FFA in cultured bag cell neurons. Parenthetically, at the same concentration used here, indomethacin blocked the actions of FMRFamide, a Cox-activating peptide, in *Aplysia* sensory neurons (Piomelli et al. 1987b).

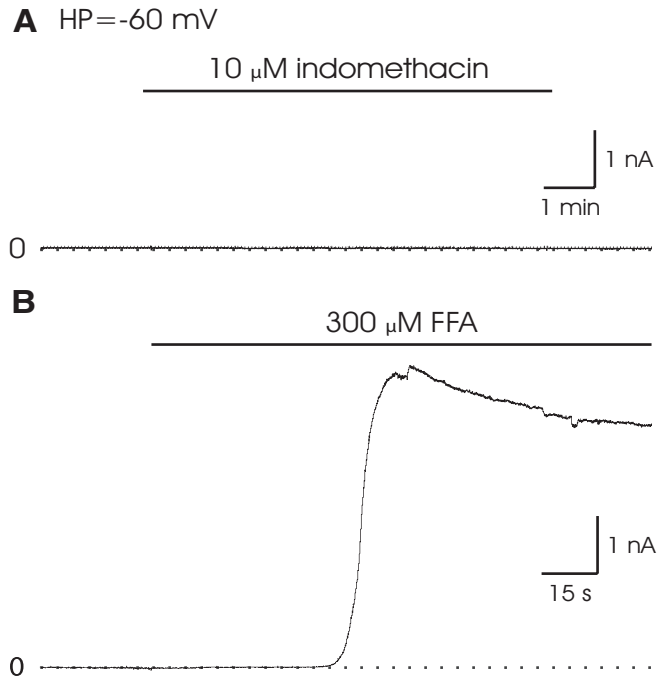

FIG. 8. Inhibition of Cox does not alter steady-state membrane current. *A*: perfusion of 10 μM indomethacin, a general Cox inhibitor, results in no change to the holding current under voltage-clamp at −60 mV in nASW (representative of $n = 7$). *B*: in the same neuron, subsequent application of 300 μM FFA results in a robust I_{out}.

The marked increase in steady-state membrane conductance associated with the FFA-induced I_{out} in cultured bag cell neurons points to channel opening. The candidate ions that could cause I_{out} are K^+ or Cl^-. The Nernst potential for Cl^- in our recording conditions is approximately −55 mV. As such, Cl^- channel opening at a holding potential of −60 mV would result in Cl^- efflux, a small inward current, and a slight, positive shift in the reversal potential of the whole cell current. On the contrary, I_{out} implicates a K^+ channel, as it is associated with a negative shift, from roughly −65 mV to nearly −80 mV, in the reversal potential. Presumably, this shift only approaches the K^+ Nernst potential (calculated to be around −100 mV) because other steady-state channels comprising the resting conductance are still open. Thus I_{out} strongly influences, but does not completely dominate, whole cell current reversal potential.

I_{out} is also sensitive to TEA, a well-recognized K^+ channel blocker (Hagiwara and Saito 1959) known to inhibit both voltage-sensitive and Ca^{2+}-activated K^+ conductances in bag cell neurons (Fink et al. 1988; Quattrocki et al. 1994). I_{out} is similar to a weakly voltage-dependent K^+ current that is activated by FMRFamide and perhaps inositol triphosphate in bag cell neurons (Fink et al. 1988; Fisher et al. 1993). I_{out} also resembles the serotonin-sensitive S-channel found in *Aplysia* sensory neurons (Shuster et al. 1991). Thus I_{out} is likely part of the resting conductance, and its gating would have profound consequences for the resting potential and excitability.

In canine jejunal smooth muscle, FFA activates a weakly voltage-sensitive outward current characterized as a K^+ channel (Farrugia et al. 1993). Similarly, FFA stimulates opening of a two-pore leak K^+ channel expressed in cell lines (Takahira et al. 2005). Furthermore, *Helix* neurons display a slow, outward current triggered by FFA (Lee et al. 1996; Shaw et al. 1995) although no information is available regarding the reversal potential, pharmacology, or voltage dependence of that current nor if it is associated with an increase in membrane conductance. Interestingly, the outward current in *Helix* is reduced by depleting the endoplasmic reticulum of Ca^{2+}. This suggests that, unlike I_{out} in bag cell neurons, the FFA-induced current in *Helix* may depend on intracellular Ca^{2+} release. The rapid onset and lack of an effect of high intracellular EGTA suggests that I_{out} is not Ca^{2+} dependent.

The FFA-evoked I_{in} in cultured bag cell neurons was also accompanied by a conductance increase and a positive shift (from approximately −70 to nearly −15 mV) in the reversal potential of the whole cell current. The current-voltage relationship during activation of I_{in} was voltage independent up to the point where it reversed, after which some outward rectification was apparent. The reversal potential is consistent with the opening of a channel that is nonselective for cations. Specifically, reversal between −40 and +20 mV is typical for channels that pass cations with a varying degree of selectivity and no overwhelming preference (Colquhoun et al. 1981; Kass et al. 1978; Partridge and Swandulla 1988; Partridge et al. 1994). Further support for I_{in} being a cation channel comes from the fact that it is blocked by Gd^{3+}. This trivalent cation is a well-established nonspecific cation channel blocker with relatively few side-effects (Chakfe and Bourque 2000; Franco and Lansman 1990; Popp et al. 1993; Yang and Sachs 1989).

Finally, it appears that I_{in} may be activated in part by FFA-induced Ca^{2+} release. Both the Ca^{2+} elevation and I_{in} required several min to fully develop; furthermore, FFA failed to evoke I_{in} when intracellular Ca^{2+} was strongly buffered with high EGTA.

If Ca^{2+}-activation is the gating mechanism for I_{in}, it is possible that one or more bag cell neuron Ca^{2+}-activated channels contributes to the conductance as a whole. While the voltage-dependent cation channel which reverses well above 0 mV is likely not I_{in} (Lupinsky and Magoski 2006; Magoski 2004; Wilson et al. 1996), the voltage-independent cation channel triggered by Ca^{2+} influx, with a reversal potential near −40 mV and a sensitivity to Gd^{3+}, may be a component (Hung and Magoski 2007). That FFA potentiated this current (Hung and Magoski 2007), despite actually inhibiting the voltage-gated Ca^{2+} current, could be due to a synergistic effect of Ca^{2+} liberation from intracellular stores and subsequent activation of I_{in}. In addition, Knox et al. (1996) reported that depletion of Ca^{2+} from the endoplasmic reticulum by thapsigargin activated a cation channel which reversed near −20 mV, was voltage-independent, and was blocked by pretreatment with BAPTA-AM. This third Ca^{2+}-activated cation channel may also contribute to I_{in}. Incidentally, it is unlikely that a possible inhibitory effect of FFA on gap junctions, as electrical synapses or hemi-channels (Harks et al. 2001; Srinivas and Spray 2003; Ye et al. 2003), is the cause of I_{in}. All of the neurons used in the present study were single cells that did not touch other neurons and had no opportunity to make electrical synapses. Regarding hemi-channels, they certainly could be present, but their block would result in a decreased whole cell conductance, rather than the increase seen with both I_{in} and I_{out}.

Ca^{2+}-activated and receptor-operated TRP cation channels from heart and arterial smooth muscle are blocked by Gd^{3+} but not FFA (Hill et al. 2006; Ohki et al. 2000). FFA also initially enhances both Ca^{2+}-activated and ligand-gated cation channels in *Helix* and hippocampal neurons (Green and Cottrell 1997; Partridge and Valenzuela 2000; Shaw et al. 1995); although once enhancement reaches a peak, a slow block then follows. Those Ca^{2+}-activated cation currents were elicited by depolarizing steps or action potentials with the enhancement thought to be due to FFA-induced release of more Ca^{2+} (Partridge and Valenzuela 2000; Shaw et al. 1995). This is similar to the bag cell neurons in that FFA can enhance cation channels by releasing Ca^{2+}. However, prior to the present study there were no reports that FFA could trigger cation channels to open at rest without depolarization-evoked Ca^{2+} influx. The bag cell neuron I_{in} and the prolonged depolarization cation current also do not show any slow block by FFA. I_{in} would influence the resting potential and, if activated by Ca^{2+} released during the afterdischarge (Fisher et al. 1994), contribute depolarizing drive to the burst.

As suggested, some of the effect of FFA on cultured bag cell neurons appears to be due to intracellular Ca^{2+} release. Our experiments involving depleting endoplasmic reticulum Ca^{2+} with CPA suggest that this Ca^{2+} may in part come from the endoplasmic reticulum. CPA blocks the Ca^{2+}-ATPase and causes the endoplasmic reticulum to lose Ca^{2+} through leak channels (Seidler et al. 1989; Tu et al. 2006). Lee et al. (1996) found that the ability of FFA to raise intracellular Ca^{2+} levels in *Helix* neurons could be largely eliminated by thapsigargin, which is functionally analogous to CPA (Thastrup et al. 1990). However, the FFA-induced Ca^{2+} increase in both a mandibular cell line and hippocampal neurons was not prevented by thapsigargin (Partridge and Valenzuela 2000; Poronnik et al. 1992). Thus as in the bag cell neurons, FFA may target other stores. Data from the present study show that the FFA Ca^{2+} response is also depressed by pretreatment with FCCP, which collapses the mitochondrial membrane potential that normally drives Ca^{2+} into the mitochondria (Collins et al. 2000; Heytler and Prichard 1962; Simpson and Russell 1996). FFA has been shown to both prevent Ca^{2+} uptake and release from liver mitochondria (Jordani et al. 2000; McDougall et al. 1988). This may be achieved through either a protonophore-like effect, similar to FCCP itself, or direct activation of the mitochondrial permeability transition pore (Jordani et al. 2000). Not surprisingly, removal of Ca^{2+} from both the endoplasmic reticulum and mitochondria, by depleting with CPA and FCCP at the same time, substantially reduced the Ca^{2+} response of the bag cell neurons to FFA.

In summary, we have provided evidence that FFA directly opens a K^+ conductance and indirectly activates a cation conductance by releasing intracellular Ca^{2+} in cultured bag cell neurons. The effect of FFA on voltage-gated Ca^{2+} current may be due to the drug acting on the channel itself or again by some Ca^{2+}-dependent process. Clearly FFA can alter the function of numerous membrane proteins; as such, the mechanism of FFA cation channel block in other systems may be related to how it alters plasma membrane, and perhaps intracellular, ion channels. In some ways, FFA has been seen as a gold-standard for cation channel blockers. However, recognizing that this drug may set off other intracellular or biophysical events, its use needs to be tempered with appropriate controls. Despite this, FFA could be employed as a tool for intentionally releasing intracellular Ca^{2+} or triggering certain currents.

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