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A Store-Operated Ca^{2+} Influx Pathway in the Bag Cell Neurons of *Aplysia*

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Kachoei, Babak A., Ronald J. Knox, Didier Uthuza, Simon Levy, Leonard K. Kaczmarek, and Neil S. Magoski. A store-operated Ca^{2+} influx pathway in the bag cell neurons of *Aplysia*. *J Neurophysiol* 96: 2688–2698, 2006. First published August 2, 2006; doi:10.1152/jn.00118.2006. Although store-operated Ca^{2+} influx has been well-studied in nonneuronal cells, an understanding of its nature in neurons remains poor. In the bag cell neurons of *Aplysia californica*, prior work has suggested that a Ca^{2+} entry pathway can be activated by Ca^{2+} store depletion. Using fura-based imaging of intracellular Ca^{2+} in cultured bag cell neurons, we now characterize this pathway as store-operated Ca^{2+} influx. In the absence of extracellular Ca^{2+}, the endoplasmic reticulum Ca^{2+}-ATPase inhibitors, cyclopiazonic acid (CPA) or thapsigargin, depleted intracellular stores and elevated intracellular free Ca^{2+}. With the subsequent addition of extracellular Ca^{2+}, a prominent Ca^{2+} influx was observed. The ryanodine receptor agonist, chloroethylphenol (CEP), also increased intracellular Ca^{2+} but did not initiate store-operated Ca^{2+} influx, despite overlap between CEP- and CPA-sensitive stores. Bafilomycin A, a vesicular H^{+}-ATPase inhibitor, liberated intracellular Ca^{2+} from acidic stores and attenuated subsequent Ca^{2+} influx, presumably by replenishing CPA-depleted stores. Store-operated Ca^{2+} influx was partially blocked by low concentrations of La^{3+} or BTP2, and strongly inhibited by either 1-[b-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole (SKF-96365) or a high concentration of Ni^{2+}. Regarding IP_3 receptor blockers, 2-aminoethyldiphenyl borate, but not xestospongin C, prevented store-operated Ca^{2+} influx. However, jasplakinolide, an actin stabilizer reported to inhibit this pathway in smooth muscle cell lines, was ineffective. The bag cell neurons initiate reproductive behavior through a prolonged afterdischarge associated with intracellular Ca^{2+} release and neuropeptide secretion. Store-operated Ca^{2+} influx may serve to replenish stores depleted during the afterdischarge or participate in the release of peptide that triggers behavior.

INTRODUCTION

Elevation of intracellular Ca^{2+} is a profoundly important signaling event, capable of producing neurotransmitter release, activating signal transduction pathways, or initiating gene expression (Hille 2001; Levitan and Kaczmarek 2002). Ca^{2+} can be released from intracellular stores, which in neuronal somata are principally the endoplasmic reticulum (Berridge 1998; Meldolesi 2001; Verkhratsky 2005), or Ca^{2+} can enter from the extracellular space through voltage-gated Ca^{2+} channels (Hille 2001; Reid et al. 2003). Because most nonneuronal/nonexcitable cells cannot employ the voltage sensitivity of Ca^{2+} channels, they gate Ca^{2+} influx through an alternative pathway known as capacitative Ca^{2+} entry or store-operated Ca^{2+} influx (Casteels and Droogmans 1981; Nilius 2003; Parekh 2003; Putney 1986). Although the mechanism is not fully defined, this pathway is activated when the depletion of Ca^{2+} from intracellular stores opens specific, Ca^{2+}-permeable channels in the plasma membrane (Nilius 2003; Parekh 2003).

In comparison to nonneuronal cells, the characterization of store-operated Ca^{2+} influx in neurons is less extensive (Putney 2003). Initially, neurons, neuroendocrine cells, and neuronal cell lines were thought to lack store-operated Ca^{2+} influx (Friel and Tsien 1992; Jackson et al. 1988; Stauderman and Pruss 1989). However, beginning with a report of Ca^{2+} entry following store depletion in PC12 cells (Clementi et al. 1992), affirmative evidence began to accumulate (Arakawa et al. 2000; Baba et al. 2003; Bouron 2000; Grudt et al. 1996; Liu and Gylfe 1997; Piper and Lucero 1999; Powis et al. 1996; Prothero et al. 2000; Tozzi et al. 2003; Usachev and Thayer 1999; Villalobos and Garcia-Sancho 1995; Zufall et al. 2000). Nevertheless, reports continue to assert that certain neurons either lack or show limited store-operated Ca^{2+} influx (Chinopoulos et al. 2004; Emptage et al. 2001; Grimaldi et al. 2001; Koizumi et al. 1999). The ongoing controversy prompted us to explore this pathway in the bag cell neurons of the marine mollusk, *Aplysia californica*—a preparation in which the regulation of intracellular Ca^{2+} has been extensively investigated (Fink et al. 1988; Jonas et al. 1997; Knox et al. 1996, 2004; Magoski et al. 2000).

Brief synaptic input to the bag cell neurons triggers an ~30-min afterdischarge, resulting in neuropeptide secretion and the initiation of egg-laying behavior (Conn and Kaczmarek 1989; Kupfermann 1967; Kupfermann and Kandel 1970; Pinsker and Dudek 1977; Rothman et al. 1983). Previously, Knox et al. (1996) used a Ca^{2+}-sensitive vibrating extracellular probe to shown that the endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin (Thastrup et al. 1990), may initiate Ca^{2+} entry in bag cell neurons. In the present study, we employ intracellular Ca^{2+} imaging to demonstrate that this indeed is a store-operated Ca^{2+} influx pathway. During an afterdischarge, Ca^{2+} enters through voltage-gated Ca^{2+} channels and is released from intracellular stores, apparently in an IP_3-dependent manner (Fink et al. 1988; Fisher et al. 1994). The bag cell neurons may require store-operated Ca^{2+} influx to replete these intracellular stores or may use this source of Ca^{2+} entry to elicit neuropeptide secretion itself.

METHODS

Animals and cell culture

Adult *A. californica* weighing 100–250 g were obtained from Marine Specimens Unlimited (San Francisco, CA) or Marinus (Long

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Beach, CA). Animals were housed in an ~300l aquarium containing continuously circulating, aerated artificial sea water (Instant Ocean, Aquarium Systems, Mentor, OH, or Kent sea salt, Kent Marine, Acworth, GA) at 14–16°C on an ~12/12 h light/dark cycle and fed Romaine lettuce three to 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), the abdominal ganglion removed and treated for 18 h at 20–22°C with neutral protease (13.33 mg/ml; 165859, Roche Diagnositics, Indianapolis, IN) 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 and the bag cell neuron clusters dissected from their surrounding connective tissue. Using a fire-polished Pasteur pipette and gentle trituration, neurons were dispersed in tcASW onto regular 35 × 10-mm polystyrene tissue culture dishes (25000, Corning, Corning, NY) or glass coverslips (No. 1; 48366045, VWR, West Chester, PA) that were coated with poly-D-lysine (1 μg/ml, 70,000–150,000 MW; P0899, Sigma-Aldrich, Oakville, ON, Canada, or St. Louis, MO) and glued to drilled out tissue culture dishes. Cultures were maintained in tcASW for 1–3 days in a 14°C incubator. Salts were obtained from Fisher (Ottawa, ON, Canada), ICN (Aurora, OH), or Sigma-Aldrich.

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 either a Picospritzer II (General Valve) or a PMI-100 pressure microinjector (Dagan, Minneapolis, MN) while simultaneously monitoring membrane potential with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA). Microelectrodes were pulled from 1.2-mm internal diameter, borosilicate glass capillaries (1B120F-4, World Precision Instruments, Sarasota, FL) 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 to be 50–100 μM. All neurons used for subsequent imaging showed resting potentials of −50 to −60 mV and displayed action potentials that overshot 0 mV after 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 Diaphot inverted microscope (Nikon, Mississauga, ON, Canada) equipped with a Nikon Fluor 10X objective (numerical aperture (NA) = 0.5) or Nikon TS100-F inverted microscope equipped with a Nikon Plan Fluor 10X 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 either a Hamamatsu C2400 (Hamamatsu, Bridgewater, NJ) or 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, if necessary, 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 interests (ROIs) defined over the neuronal somata prior to the start of the experiment. The ratio was then either recorded simply as 340/380 to reflect free intracellular Ca^{2+} or used to calculate the actual free intracellular Ca^{2+} from the relationship, $[Ca^{2+}] = K_d \cdot Q(R - R_{min})/R_{max} - R$ (Grynkiewicz et al. 1985). R_{min}, R_{max}, and Q were determined in intact bag cell neurons by applying 1–10 μM digitonin (D-8449, Molecular Probes, Eugene, OR) under Ca^{2+}-free conditions followed by perfusion with saline containing a saturating amount of Ca^{2+} (11 mM). The constant Q was determined from the ratio of 380 nm evoked fura PE3 fluorescence in Ca^{2+}-free ASW and 11 mM Ca^{2+}-containing normal ASW (nASW). Values for R_{min}, R_{max}, and Q ranged from 0.11 to 0.33, 5.1–7.5, and 42.6–50, respectively, whereas the K_d was 204 nM (from Vorndran et al. 1995). 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 (composition as per tcASW but with the glucose, penicillin, and streptomycin omitted) and Ca^{2+}-free ASW (composition as per nASW but with $CaCl_2$ omitted and 0.5 mM EGTA added).

Membrane potential recordings

An Axoclamp 2B amplifier (Axon Instruments) in bridge mode was used to measure membrane potential. Current-clamp recordings were made using sharp microelectrodes (glass as per dye injection) with a resistance of 5–10 MΩ when filled with 2 M K-acetate (supplemented with 100 mM KCl and 10 mM HEPES; pH = 7.3 with KOH). Voltage was low-pass filtered at 3 kHz using the Axoclamp 2B Bessel filter and acquired at a sampling rate of 100 Hz with an IBM-compatible personal computer, a Digidata 1300 A/D converter (Axon Instruments), and the Clampex acquisition program of pCLAMP (version 8.0; Axon Instruments).

Drug application and reagents

Drug application or solution exchanges were accomplished by manual perfusion using a plastic transfer pipette to exchange the bath (tissue culture dish) solution. Complete exchange of the bath could be achieved in <30 s. 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 neurons. 2-aminoethyldiphenyl borate (2-APB; 100065, Calbiochem, San Diego, CA), bafilomycin A (B1793, Sigma-Aldrich), BTP2 (203890, Calbiochem), 4-chloro-3-ethylphenol (CEP; 279552, Sigma-Aldrich), cyclopiazonic acid (CPA; C1530, Sigma-Aldrich or 239805, Calbiochem), 1-[b-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole (SKF 96365; 567310, Calbiochem or S7809, Sigma-Aldrich), thapsigargin (T3250, Sigma-Aldrich), and xestospongin C (CA409, Cedarlane, Hornby, ON, Canada) all required DMSO (BP231, Fisher) as a vehicle, whereas jasplakinolide (420107, Calbiochem) required methanol (A412, Fisher). The maximal final concentration of DMSO and methanol was 0.01 μM and 0.01%, respectively, which in control experiments had no effect on intracellular Ca^{2+}. $LaCl_3$ (L4131, Sigma-Aldrich) and $NiCl_2$ (N6136, Sigma-Aldrich) were dissolved as a stock in water or to the final desired concentration in saline.

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Analysis

Origin (version 7, OriginLab Corporation, Northampton, MA) was used to import and plot ImageMaster Pro files as line graphs. For display, intracellular Ca^{2+} concentrations or the 340/380 ratios were averaged and presented as the means ± SE versus time. Analysis used steady-state changes acquired by taking the average value, by eye or with adjacent-averaging, from regions that had reached steady state for 5–10 min in plots of individual experiments. Statistics were performed on percent change values using Instat (version 3.0, GraphPad Software, San Diego, CA). The Kolmogorov-Smirnov method was used to test data sets for normality. A standard ANOVA with the Dunnett's multiple comparisons test was used to test for differences between multiple means. In a few cases, Student's paired or unpaired *t*-test was used to test for differences between two means. Data were considered significantly different if the two-tailed *P* value was <0.05.

RESULTS

Intracellular Ca^{2+} store depletion activates a Ca^{2+} influx pathway in cultured bag cell neurons

To determine if Ca^{2+} store depletion can initiate a Ca^{2+} influx pathway, cultured bag cell neurons were bathed in Ca^{2+}-free ASW and exposed to agents that liberate intracellular Ca^{2+}. The smooth endoplasmic reticulum Ca^{2+} pump inhibitor, CPA (10–50 μM) (Seidler et al. 1989), depleted intracellular stores and caused Ca^{2+} to rise quickly (Fig. 1*A*; $n = 12$). Despite the continued presence of CPA, Ca^{2+} levels recovered to near-control levels, most likely attributable to active and passive removal of Ca^{2+} from the intracellular to the extracellular compartment (Clapham 1995; Knox et al. 1996; Meldolesi 2001; Verkhratsky 2005). In separate experiments, the subsequent addition of extracellular Ca^{2+} by exchanging the Ca^{2+}-free ASW for nASW initiated a marked and rapid rise in intracellular Ca^{2+} but only in those neurons depleted with CPA and not those merely exposed to Ca^{2+}-free ASW alone (Fig. 1*B*; $n = 44$ versus 11). This suggested that depletion of intracellular Ca^{2+} stores activates a plasma membrane Ca^{2+} entry pathway. Although this pathway is presumably open during depletion in Ca^{2+}-free conditions, it cannot be detected until extracellular Ca^{2+} is added and Ca^{2+} begins to flow back into the neurons. Similar results were achieved with 2–3 μM of the irreversible, smooth endoplasmic reticulum Ca^{2+} pump inhibitor, thapsigargin (Thastrup et al. 1990) (Fig. 1*C*; $n = 15$). On average, addition of extracellular Ca^{2+} after depletion with CPA resulted in an ~47% increase in intracellular Ca^{2+} that was statistically different from the ~25% increase observed following thapsigargin-induced depletion (Fig. 6; 2nd vs. 1st bar).

It is possible that the store-operated pathway depolarizes the neurons to such an extent that voltage-gated Ca^{2+} channels are activated. This would contaminate the assay with an additional Ca^{2+} influx source. To resolve this, the membrane potential of bag cell neurons was recorded during the introduction of extracellular Ca^{2+} after depletion. After depletion with CPA in Ca^{2+}-free ASW, exchange to Ca^{2+}-containing nASW resulted in only a small depolarization of 8.7 ± 4.3 mV (Fig. 1D; $n = 6$). In Ca^{2+}-free ASW plus CPA, the actual membrane potential was −52.8 ± 6.3 mV, whereas in nASW plus CPA, it depolarized to −45.6 ± 3.9 mV (not significant; Student's paired *t*-test). Such a change would fail to bring the membrane potential near the threshold for activation of the voltage-gated Ca^{2+} current in these neurons (−30 to −25 mV) (Kaczmarek and Strumwasser 1984; Knox et al. 1996; A. Y. Hung and N. S. Magoski, unpublished data).

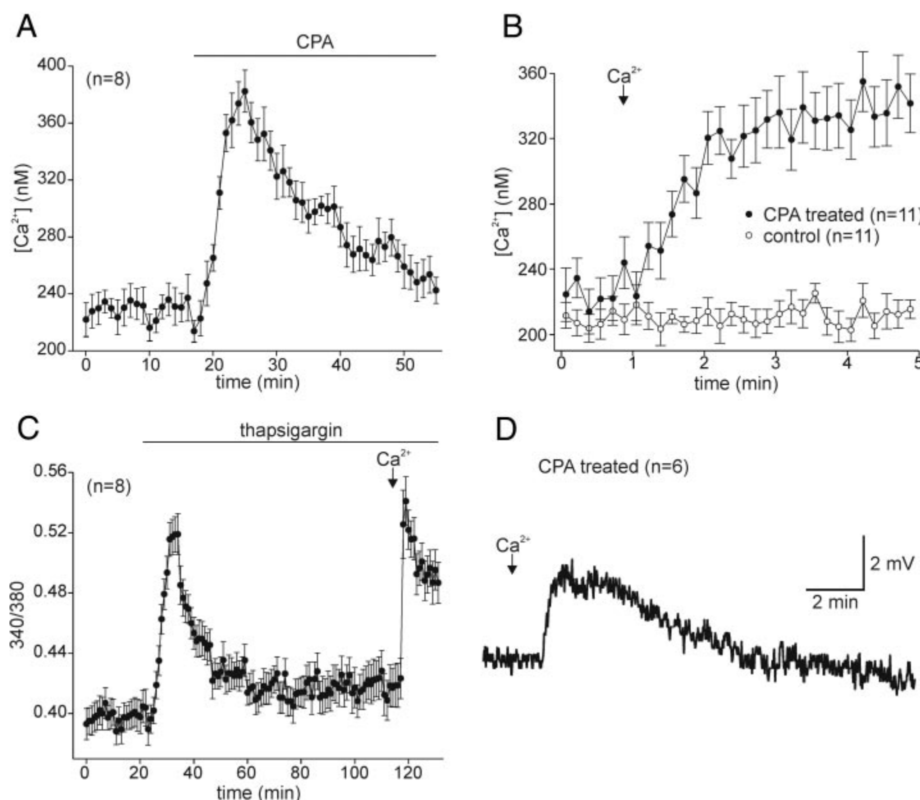

FIG. 1. Depletion of cultured bag cell neuron intracellular Ca^{2+} stores initiates a store-operated Ca^{2+} influx pathway. *A*: addition of 10 μM of the smooth endoplasmic reticulum Ca^{2+} pump inhibitor, cyclopiazonic acid (CPA), causes Ca^{2+} store depletion and elicits a large, rapid Ca^{2+} rise that subsequently recovers ($n = 8$; representative of 12 in total). *B*: exchanging the Ca^{2+}-free ASW for Ca^{2+}-containing nASW (↓), results in a rapid elevation of intracellular Ca^{2+} in neurons depleted with CPA ($n = 11$; representative of 44 in total) but not in neurons simply maintained in Ca^{2+}-free ASW ($n = 11$). The CPA-treated neurons were exposed to the drug for ~60 min prior to the addition of nASW. *C*: similarly, 3 μM of thapsigargin, a different smooth endoplasmic reticulum Ca^{2+} pump inhibitor, also depletes the Ca^{2+} stores of bag cell neurons bathed in Ca^{2+}-free ASW. The introduction of extracellular Ca^{2+} again initiates an accelerated rise in intracellular Ca^{2+} ($n = 8$; representative of 15 in total). *D*: sharp-electrode current clamp recording of membrane potential from a cultured bag cell neuron bathed in Ca^{2+}-free ASW plus 10 μM CPA for 30 min. Delivery of Ca^{2+}-containing nASW elicits a small depolarization that recovers to baseline in ~5 min ($n = 6$).

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Activating ryanodine receptors does not initiate the store-operated Ca^{2+} influx pathway

Although CPA and thapsigargin liberate Ca^{2+} by blocking Ca^{2+} pumps on the endoplasmic reticulum, an alternate means is to open the Ca^{2+}-permeable channels within the store. In Ca^{2+}-free ASW, 100 μM of the ryanodine receptor agonist, CEP (Zorzato et al. 1993), rapidly elevated intracellular Ca^{2+}, which then returned to near baseline levels even with maintained exposure to the agonist (Fig. 2*A*; $n = 15$). Subsequent delivery of extracellular Ca^{2+} caused, on average, intracellular Ca^{2+} to rise by only ~5%, which was significantly different from the influx that occurred with CPA (Fig. 6; 3rd vs. 1st bar). When CPA was applied after CEP in Ca^{2+}-free ASW, there was a small but detectable Ca^{2+} elevation as well as obvious store-operated Ca^{2+} influx on return to Ca^{2+}-containing nASW (Fig. 2*B*; $n = 8$). Overall, addition of extracellular Ca^{2+} to neurons treated with CEP followed by CPA resulted in an ~55% increase in intracellular Ca^{2+} that was not significantly different from that seen after CPA alone (Fig. 6; 4th vs. 1st bar). CEP was chosen as a ryanodine receptor agonist over caffeine (Rousseau et al. 1988; Weber 1968) or ryanodine (Meissner 1985) because we have found that the former has a number of nonspecific effects on bag cell neuron ion channels, whereas the latter is not an efficacious agonist of the bag cell neuron Ca^{2+} release channel—perhaps because of the high salt conditions (N. S. Magoski, R. J. Knox, and L. K. Kaczmarek, unpublished observation).

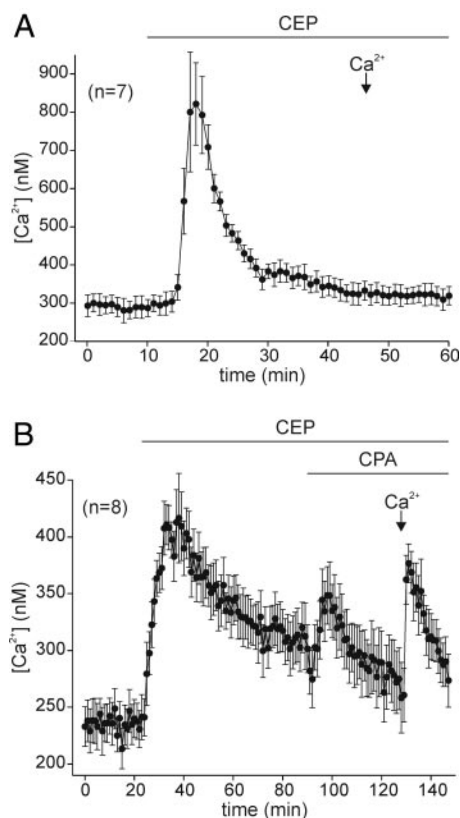

FIG. 2. A ryanodine receptor agonist elevates intracellular Ca^{2+} but does not cause store-operated Ca^{2+} influx. *A*: in Ca^{2+}-free ASW, 100 μM of chloroethylphenol (CEP), a ryanodine receptor agonist, causes a rapid increase in intracellular Ca^{2+} followed by almost complete recovery. However, when extracellular Ca^{2+} is introduced on exchange to nASW, intracellular Ca^{2+} remains unchanged ($n = 7$; representative of 15 in total). *B*: prior opening of ryanodine receptors by 100 μM CEP reduces the Ca^{2+} elevation produced by the application of 10 μM CPA in Ca^{2+}-free ASW. Nevertheless, after application of CEP and CPA, store-operated Ca^{2+} influx elicited by exchange to Ca^{2+}-containing nASW remains intact ($n = 8$).

Ca^{2+} liberated from acidic stores replenishes stores depleted with CPA

The acidic compartment is a bag cell neuron Ca^{2+} store that has yet to be investigated. Bafilomycin A, a vacuolar H^+-ATPase inhibitor (Bowman et al. 1988), caused bag cell neuron intracellular Ca^{2+} to slowly rise in Ca^{2+}-free ASW when applied at 50 nM (Fig. 3*A*; $n = 6$). The source was designated an acidic store because Ca^{2+} was liberated after the bafilomycin-A-induced collapse of its H^+ gradient, which is presumably required for maintaining Ca^{2+} uptake (Christensen et al. 2002; Dunn et al. 1994; Goncalves et al. 1999; Ohsumi and Anraku 1983). The time to onset of the bafilomycin-A-induced elevation in intracellular Ca^{2+} was shorter if the neurons were first exposed to CPA (Fig. 3*B*; $n = 15$); however, the mean change for bafilomycin A alone (39.7 ± 4.65% increase) versus CPA then bafilomycin A (56.6 ± 4.8% increase) only approached significance ($P < 0.06$; Student's unpaired *t*-test). Wash of the bafilomycin A + CPA resulted in intracellular Ca^{2+} recovering to near CPA-depleted levels, whereas subsequent exchange to Ca^{2+}-containing nASW initiated some store-operated Ca^{2+} influx, although it was very muted (Fig. 3, *B* and *C*). Compared with responses after depletion in CPA alone, the amplitude of the response was small and the time to onset was markedly slower (compare Figs. 1*B* with 3*C*). At only an ~8% increase, the average Ca^{2+} influx after introduction of extracellular Ca^{2+} to bafilomycin A + CPA treated neurons was significantly different from that after CPA alone (Fig. 6; 5th vs. 1st bar).

Store-operated Ca^{2+} influx pathway is sensitive to La^{3+}, Ni^{2+}, SKF 96365, and BTP2

Depleting intracellular stores clearly initiates Ca^{2+} influx in bag cell neurons. However, to better compare this route of Ca^{2+} entry with similar pathways in other neuronal and non-neuronal cells, it was important to learn more about its pharmacological properties. Store-operated Ca^{2+} influx is often blocked by tri- or divalent metals such as La^{3+} and Ni^{2+} (Gore et al. 2004; Hoth and Penner 1993; Ross and Cahalan 1995). With 100 μM La^{3+} in the bath, addition of extracellular Ca^{2+} to CPA-depleted bag cell neurons brought about a rise in intracellular Ca^{2+} that was more modest in comparison to control (Fig. 4*A*; $n = 23$). Although this block was by no means complete, the difference between the mean change in intracellular Ca^{2+} in La^{3+} (~23% increase) was significantly different from that in control (~47% increase; Fig. 6; 6th vs. 1st bar). When the same experiment was performed using 100 μM Ni^{2+}, no block of the Ca^{2+} influx pathway was seen (Fig. 4*B*; $n = 10$), although increasing the concentration of Ni^{2+} to 10 mM quite effectively inhibited the increase in intracellular Ca^{2+} normally observed with the addition of Ca^{2+}-containing nASW (Fig. 4*C*; $n = 13$). The average change in intracellular Ca^{2+} after the introduction of extracellular Ca^{2+} to neurons treated with CPA +100 μM Ni^{2+} was an ~40% increase, which was not significantly different from CPA alone (Fig. 6; 7th vs. 1st bar). Conversely, there was only a mean ~10%

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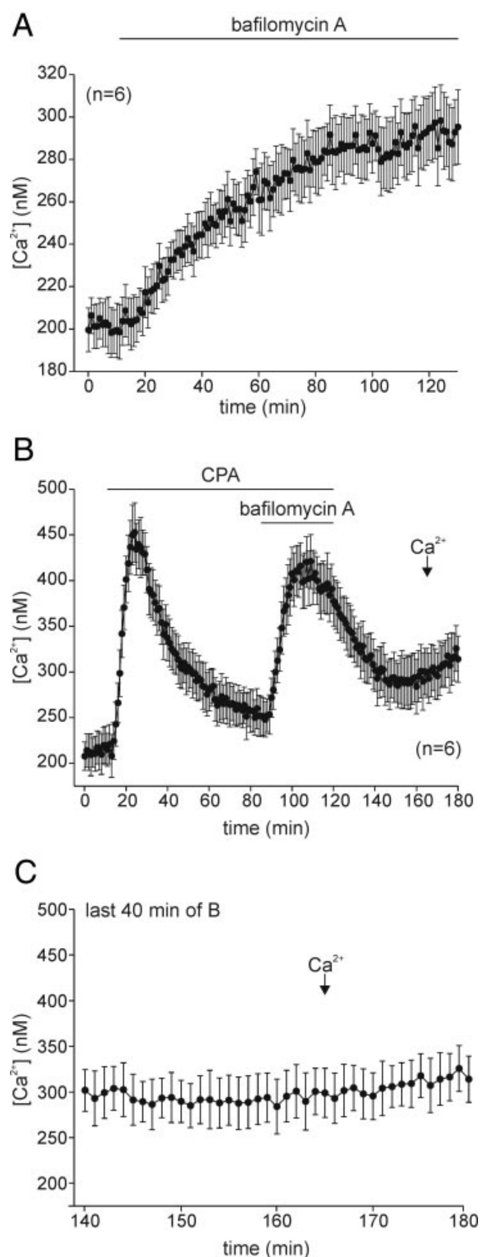

FIG. 3. H^+-ATPase inhibitor elevates intracellular Ca^{2+} and replenishes depleted Ca^{2+} stores. *A*: under Ca^{2+}-free conditions, a gradual elevation of intracellular Ca^{2+} is induced by 50 nM of the vacuolar H^+-ATPase inhibitor, bafilomycin A (n = 6). *B*: Ca^{2+} release from acidic stores partially replenishes previously depleted stores. Addition of 50 nM bafilomycin A, after depletion by 20 μM CPA, causes intracellular Ca^{2+} to rise at a rapid rate in Ca^{2+}-free ASW (compare with *A*). On wash of both bafilomycin A and CPA, Ca^{2+} levels show partial recovery; moreover, exchange to Ca^{2+}-containing nASW produces only a slow, attenuated elevation of intracellular Ca^{2+} (n = 6; representative of 15 in total). *C*: The last 40 min of *B* on an expanded time scale. The record begins just after Ca^{2+} levels have recovered after washout of the drugs. If at all, extracellular Ca^{2+} produces a small, slow elevation of intracellular Ca^{2+}.

increase in intracellular Ca^{2+} for CPA +10 mM Ni^{2+}, and this value readily reached the level of significance when compared with neurons treated with just CPA (Fig. 6; 8th vs. 1st bar).

In addition to tri- or divalent metals, an imidazole known as SKF-96365 is commonly used to block store-operated Ca^{2+} influx (Cabello and Schilling 1993; Daly et al. 1995, Doi et al. 2000; Fasolato et al. 1990; Merritt et al. 1990). The Ca^{2+} entry route initiated by store depletion in bag cell neurons was antagonized by 20 μM SKF 96365 (Fig. 4*D*; n = 15). The mean change in intracellular Ca^{2+} after exchange to Ca^{2+}-containing nASW for those neurons exposed to CPA + SKF 96365 was an ~8% increase, and this was significantly different from the Ca^{2+} elevation elicited in neurons subjected to only CPA (Fig. 6; 9th vs. 1st bar). Recently, a pyrazole derivative designated as BTP2 was found to block both store-operated Ca^{2+} influx and depletion-activated current in Jurkat T-lymphocytes (Ishikawa et al. 2003; Zitt et al. 2004). For bag cell neurons, 1 μM BTP2 inhibited store-operated Ca^{2+} influx (Fig. 4E; n = 12). In neurons exposed to CPA + BTP2 there was an ~25% elevation in intracellular Ca^{2+} after exchange to nASW, which was significantly different from the approximately 47% increase seen in neurons given only CPA (Fig. 6; 10th vs. 1st bar).

Neither IP_3 receptors nor microfilaments likely play a role in store-operated Ca^{2+} influx

Ma et al. (2000) reported that inhibition of IP_3 receptors by the membrane-permeable antagonist, 2-APB (Maruyama et al. 1997), prevents store-operated Ca^{2+} influx. However, subsequent work has suggested that 2-APB is in fact a direct inhibitor of store-operated channels (Baba et al. 2003; Braun et al. 2001; Cordova et al. 2003; Prakriya and Lewis 2001; Tozzi et al. 2003). Therefore, we felt it necessary to test the ability of this agent to block Ca^{2+} entry after store depletion in bag cell neurons. For bag cell neurons that had been depleted with CPA in Ca^{2+}-free ASW, the application of 100 μM 2-APB itself produced an elevation in intracellular Ca^{2+}; moreover, 2-APB quite effectively prevented store-operated Ca^{2+} influx when extracellular Ca^{2+} was introduced with nASW (Fig. 5*A*; n = 17). On average, there was only an ~9% increase in intracellular Ca^{2+} when Ca^{2+}-containing nASW was delivered to neurons treated with CPA +2-APB, which was significantly different from the ~47% increase seen in the CPA alone treated neurons (Fig. 6; 11th vs. 1st bar). Perhaps on account of its antagonistic actions or because it simply is not a true depleting agent, store-operated Ca^{2+} influx was not initiated following release of intracellular Ca^{2+} by 2-APB alone (Fig. 6; 12th vs. 1st bar). The release of intracellular Ca^{2+} by 2-APB was not altogether unexpected. In their initial report that 2-APB could inhibit IP_3 receptors with an IC_{50} of 42 μM, Maruyama et al. (1997) noted that >90 μM, it also caused Ca^{2+} release. Because 2-APB is known to inhibit both IP_3 receptors and store-operated channels, we tested xestospongin C, an alkaloid IP_3 receptor antagonist that is thought to attenuate store-operated Ca^{2+} influx by interfering with the coupling between depletion and activation of the pathway (Gafni et al. 1997; Kiselyov et al. 1998; Ma et al. 2000). Despite the effectiveness of 2-APB, 15 μM xestospongin C did not alter store-operated influx in CPA-depleted bag cell neurons (Fig. 5*B*; n = 17). Overall, intracellular Ca^{2+} went up by ~57% when Ca^{2+}-containing nASW was introduced to neurons bathed in CPA + xestospongin C. This elevation was not significantly different from that observed in neurons bathed in CPA alone (Fig. 6; 13th vs. 1st bar).

Both Yao et al. (1999) and Patterson et al. (1999) provided evidence that store-operated Ca^{2+} influx in *Xenpous* oocytes or mammalian cell lines was initiated by secretion or trafficking of a signal from the endoplasmic reticulum to the plasma membrane. In the case of cell lines, influx was blocked fol-

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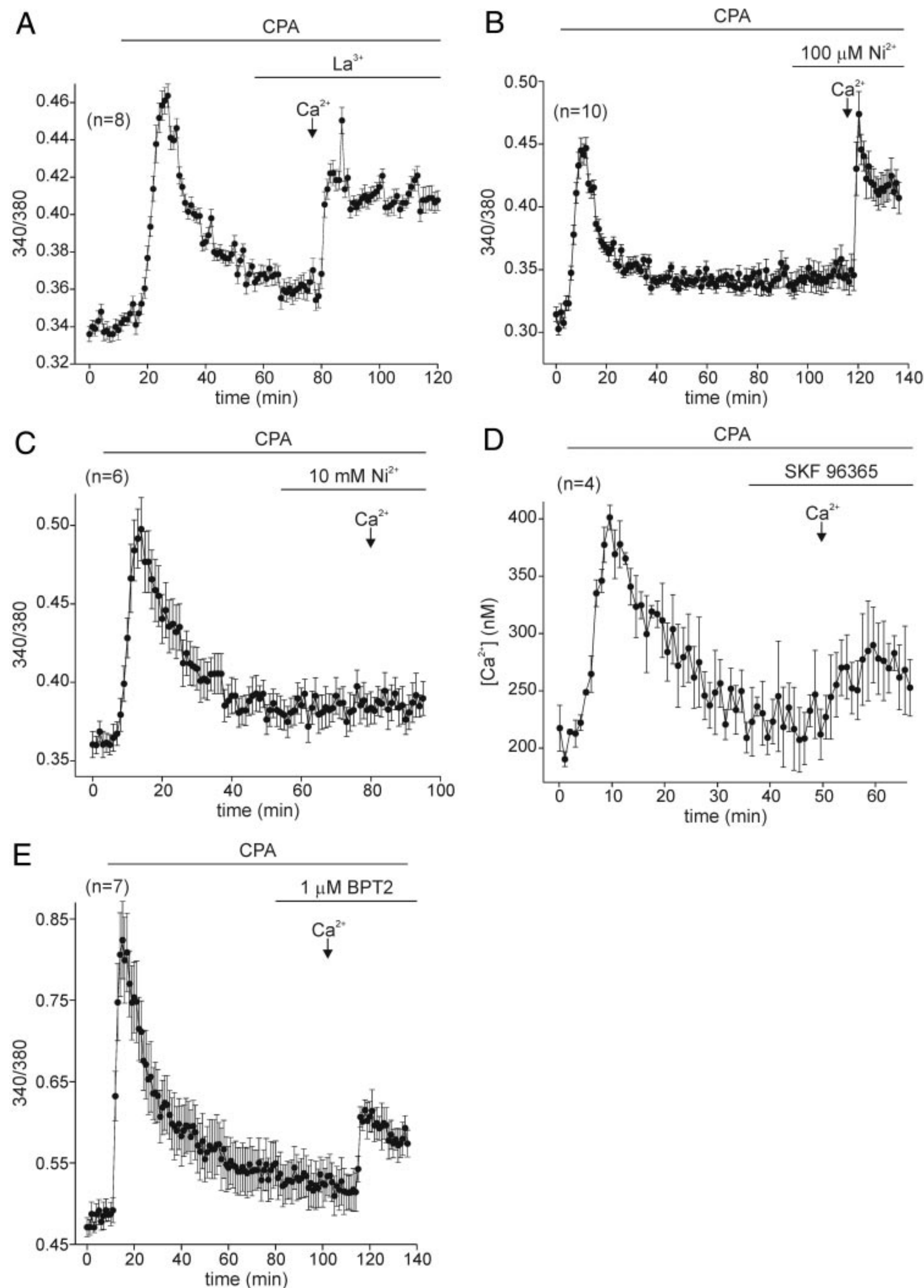

FIG. 4. Block of store-operated Ca^{2+} influx. *A*: after depletion in Ca^{2+}-free ASW with 10 μM CPA, introduction of extracellular Ca^{2+} in the presence of 100 μM La^{3+} results in attenuated store-operated Ca^{2+} influx (n = 8; representative of 23 in total). *B*: same concentration of Ni^{2+} does not prevent store-operated Ca^{2+} influx induced by prior depletion (n = 10). *C*: however, when the concentration of Ni^{2+} is increased 100-fold, it results in complete block of store-operated Ca^{2+} influx initiated by depleting stores with 10 μM CPA in Ca^{2+}-free ASW (n = 6; representative of 13 in total). *D*: Ca^{2+} stores are first depleted with 50 μM CPA in Ca^{2+}-free ASW. With the subsequent addition of 20 μM 1-[b-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole (SKF 96365), a common antagonist of store-operated Ca^{2+} influx, the exchange to nASW elicits only a small Ca^{2+} elevation (n = 4; representative of 15 in total). *E*: similarly, after depletion by 20 μM CPA in Ca^{2+}-free ASW, the presence of a different store-operated Ca^{2+} influx blocker, BTP2, results in only a modest Ca^{2+} increase when Ca^{2+}-containing nASW is introduced (n = 7; representative of 12 in total).

lowing microfilament stabilization with the membrane-permeable actin polymerizer, jasplakinolide (Bubb et al. 1994; Patterson et al. 1999). However, when 1 μM jasplakinolide was applied to bag cell neurons, store-operated Ca^{2+} influx was not prevented (Fig. 5*C*; n = 17). There was no significant difference between the ~35% mean change in intracellular Ca^{2+} after introduction of extracellular Ca^{2+} to neurons exposed to CPA + jasplakinolide, and the ~47% increase for neurons treated with only CPA (Fig. 6; 14th vs. 1st bar).

DISCUSSION

Treatment with CPA or thapsigargin in Ca^{2+}-free saline, followed by the introduction of extracellular Ca^{2+}, elevates bag cell neuron intracellular Ca^{2+} in a manner consistent with store-operated Ca^{2+} influx. Store-operated Ca^{2+} influx will not occur unless depletion opens the pathway *and* extracellular Ca^{2+} is present to enter the neurons. CPA and thapsigargin deplete Ca^{2+} by inhibiting the endoplasmic reticulum Ca^{2+}-ATPase (Seidler et al. 1989; Thastrup et al. 1990). Ca^{2+} then leaks out of the organelle and is removed to the extracellular space by plasma membrane exchangers or pumps (Clapham 1995; Meldolesi 2001; Verkhratsky 2005). It is unlikely that Ca^{2+}-free saline itself triggers influx given that without prior depletion, no change in intracellular Ca^{2+} is observed on delivery of extracellular Ca^{2+}. These results extend the findings of Knox et al. (1996), who detected Ca^{2+} entry into bag

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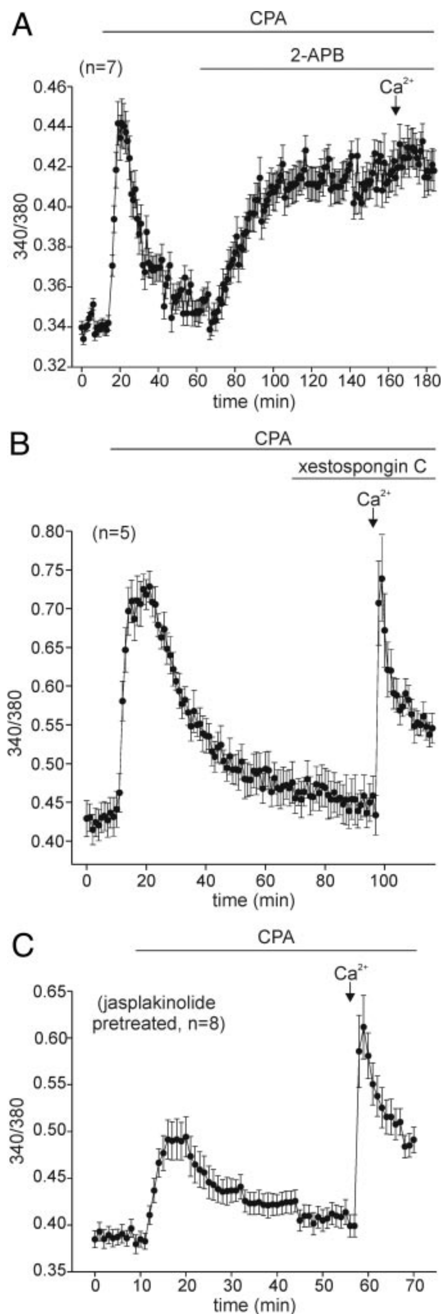

FIG. 5. Store-operated Ca^{2+} influx likely does not invovle IP_3 receptors or microfilaments. *A*: after Ca^{2+} store depletion with 10 μM CPA, the introduction of 100 μM 2-aminoethyldiphenyl borate (2-APB), a purported IP_3 receptor antagonist, elicits a 2nd rise in intracellular Ca^{2+}. When the Ca^{2+}-free ASW is exchanged to nASW, the store-operated Ca^{2+} influx is absent (n = 7; representative of 17 in total). *B*: application of 15 μM xestospongin C, a different IP_3 receptor blocker, to neurons depleted with 20 μM CPA, neither alters the intracellular Ca^{2+} nor affects store-operated Ca^{2+} influx (n = 5; representative of 17 in total). *C*: When neurons are first pretreated with 1 μM of the microfilament stabilizer, jasplakinolide, the store-operated Ca^{2+} influx produced by depletion with 10 μM CPA is unaltered (n = 8; representative of 17 in total).

cell neurons with a Ca^{2+}-sensitive vibrating extracellular probe after application of thapsigargin in the presence of extracellular Ca^{2+}. However, they did not determine if store depletion itself initiates Ca^{2+} entry nor did they demonstrate sensitivity to store-operated channel antagonists.

Both neuronal and nonneuronal store-operated channels are sensitive to certain di- and trivalent metals as well as SKF 96365, BTP2, and 2-APB. La^{3+} is the most potent metal, producing near-complete block at 0.1–1 mM (Baba et al. 2003; Bouron 2000; Gore et al. 2004; Grudt et al. 1996; Hoth and Penner 1993; Lepple-Wienhues and Cahalan 1996; Liu and Gylfe 1997; Prothero et al. 2000; Ross and Cahalan 1995). Ni^{2+}, the other metal used here, is less effective and requires 5–10 mM for substantial inhibition (Arakawa et al. 2000; Hoth and Penner 1993; Prothero et al. 2000; Trepakova et al. 2001; Usachev and Thayer 1999; Villalobos and Garcia-Sancho 1995). Regarding SKF 96365, Merritt et al. (1990) first proposed it as a blocker of store-operated Ca^{2+} influx, and although not absolutely specific, inhibition by 10–100 μM remains a key indicator of this pathway (Arakawa et al. 2000; Cabello and Schilling 1993; Daly et al. 1995; Grudt et al. 1996; Prakriya and Lewis 2003). The later developed BTP2 will completely antagonize store-operated influx after short-term incubation at 10 μM, whereas 1 μM produces a less potent reduction (Ishikawa et al. 2003). Finally, 10–100 μM 2-APB eliminates store-operated influx (Baba et al. 2003; Braun et al. 2001; Cordova et al. 2003; Prakriya and Lewis 2001; Tozzi et al. 2003). Accordingly, the profile of the bag cell neuron Ca^{2+} elevation, i.e., partial block by 100 μM La^{3+} and 1 μM BTP2, as well as near-complete block by 10 mM Ni^{2+}, 20 μM SKF 96365, or 100 μM 2-APB, is consistent with store-operated Ca^{2+} influx channels.

There are two categories of store-operated Ca^{2+} influx channels. One, Ca^{2+} release-activated Ca^{2+} (CRAC) channels, which are highly Ca^{2+} selective, very-small-conductance (estimated ~20 fS) channels (Hoth and Penner 1993; Prakriya and Lewis 2003; Zweifach and Lewis 1993). Two, Ca^{2+}-permeable, larger-conductance (tens of pS), nonselective cation chan-

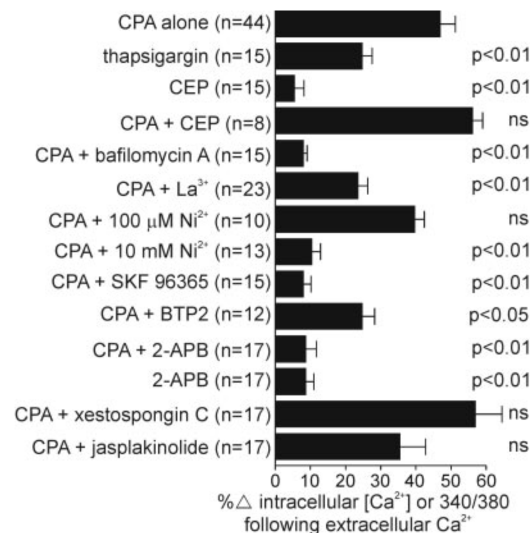

FIG. 6. Summary of store-operated Ca^{2+} influx in bag cell neurons. The ordinate lists various treatment conditions, with the n values of the total number of neurons corresponding to both those given in the text and those given in the figure legends as "representative of n in total." The abscissa is an index of store-operated Ca^{2+} influx as the percent change in either the intracellular Ca^{2+} concentration or the 340/380 ratio following the addition of extracellular Ca^{2+}. All data sets passed the test for normality using the Kolmogorov-Smirnov method. The P values on the right represent the outcome of a Dunnett's multiple comparisons test following a standard ANOVA. Comparisons were made between CPA alone and each subsequent condition.

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nels (Albert and Large 2002; Curtis and Scholfield 2001; Su et al. 2001; Trepakova et al. 2001). Some of the latter belong to the transient receptor potential channel family originally cloned from *Drosophila* (Beech et al. 2003; Kiselyov et al. 1998; Ma et al. 2000; Nilius 2003; Petersen et al. 1995; Philipp et al. 1996; Tozzi et al. 2003; Zitt et al. 1996). Recently, a membrane-spanning protein, termed Orai1 or CRACM1, has been identified as an fundamental constituent or regulator of store-operated Ca^{2+} influx channels (Feske et al. 2006; Vig et al. 2006). The channel-type mediating bag cell neuron store-operated Ca^{2+} influx is currently unknown, although the modest depolarization seen with extracellular Ca^{2+} following depletion may be due to its opening. Alternatively, store-operated Ca^{2+} influx could cause depolarization by triggering one of the bag cell neuron Ca^{2+}-activated cation channels (Knox et al. 1996; Lupinsky and Magoski 2006). Nevertheless, the source of Ca^{2+} during influx appears to be solely from the store-operated pathway, given that the depolarization is too small to activate voltage-gated Ca^{2+} current (−30 to −25 mV threshold) (Kaczmarek and Strumwasser 1984; Knox et al. 1996; A. Y. Hung and N. S. Magoski, unpublished data).

Models for depletion-activation of store-operated channels include diffusional coupling of an unknown small-molecule released from stores to stimulate channels (Randriamampita and Tsien 1993), trafficking or secretion coupling of channels or channel regulators, such a STIM1, to the plasma membrane (Patterson et al. 1999; Spassova et al. 2006Yao et al. 1999; Zhang et al. 2005), and conformational coupling of IP$_3$ receptors through a physical link with channels (Kiselyov et al. 1998; Ma et al. 2000). In smooth muscle cells and platelets, secretion coupling of store-operated Ca^{2+} influx is prevented by jasplakinolide-induced microfilament stabilization (Patterson et al. 1999; Rosado et al. 2004). However, this is not the case for epithelia or thyroid cells (Abeele et al. 2004; Gratschev et al. 2004) or, as shown in the current study, the bag cell neurons. The ineffectiveness of jasplakinolide cannot be explained by species differences in pharmacology because jasplakinolide and its membrane-impermeant analogue, phalloidin, are effective cytoskeletal stabilizers in *Aplysia* and *Drosophila* (Forscher et al. 1992; Tilney et al. 2004). Thus, trafficking or secretion coupling are unlikely to contribute to store-operated Ca^{2+} influx in bag cell neurons. For conformational coupling, a hallmark is the block of store-operated Ca^{2+} influx by IP$_3$ receptor antagonists, such as 2-APB and xestospongin C (Kiselyov et al. 1998; Ma et al. 2000; Maruyama et al. 1997). For the present study, 2-APB inhibited store-operated Ca^{2+} influx, yet xestospongin C was ineffective. Given that 2-APB also blocks store-operated channels directly (Prakriya and Lewis 2001), the parsimonious conclusion would be that diffusional coupling, rather than conformational coupling via IP$_3$ receptors, is the mechanism in bag cell neurons.

The overlap between stores with CPA-sensitive Ca^{2+} pumps and stores with CEP-sensitive ryanodine receptors influences bag cell neuron store-operated Ca^{2+} influx. Although, store-operated Ca^{2+} influx can be initiated by ryanodine receptors in DRG neurons and smooth muscle (Curtis and Scholfield 2001; Usachev and Thayer 1999), CEP fails to activate this pathway in bag cell neurons despite causing Ca^{2+} release. The failure to trigger influx may be due to CEP not preventing Ca^{2+} uptake, which leaves the Ca^{2+} in the stores at a lowered but not depleted level. Interaction also occurs between acidic stores and the endoplasmic reticulum. Acidic stores sequester Ca^{2+} via a Ca^{2+}/H^{+} exchanger driven by the H^{+} gradient (Christensen et al. 2002; Dunn et al. 1994; Goncalves et al. 1999; Ohsumi and Anraku 1983). Inhibition of bag cell neuron vacuolar H^{+}-ATPases with bafilomycin A (Bowman et al. 1988) initiates a slow rise in intracellular Ca^{2+}. Because this rise is accelerated by CPA, the endoplasmic reticulum presumably takes up some of the Ca^{2+} released by bafilomycin A. Moreover, the Ca^{2+} liberated from acidic stores appears to replenish the CPA-sensitive stores, given that store-operated influx is attenuated on return to Ca^{2+}-containing saline. Although mitochondrial Ca^{2+} uptake is known to promote CRAC channel activation (Gilabert and Parekh 2000; Glitsch et al. 2002), this is the first report, to our knowledge, of acidic stores regulating store-operated Ca^{2+} influx. Vacuolar H^{+}-ATPases are not localized to mitochondria but are found on a number of organelles, including vesicles (Calakos and Scheller 1996; Nelson 1992). Vesicles have low pH with high Ca^{2+}, and if the H^{+}-pump is blocked, Ca^{2+} will leak into the cytosol (Floor et al. 1990; Goncalves et al. 1999; Grohovaz et al. 1996; Scheenen et al. 1998; Thirion et al. 1995). Jonas et al. (1997) demonstrated that insulin triggers bag cell neuron peptide secretion by releasing Ca^{2+} from a nonendoplasmic reticulum/nonmitochondrial store that likely represents vesicles or granules.

Store-operated Ca^{2+} influx is universal in nonneuronal cells (Prakriya and Lewis 2003; Putney 2003), but for neurons it clearly varies between species and cell type. Although not detected in bullfrog sympathetic neurons or rat hippocampal and cortical neuron cultures (Chinopoulos et al. 2004; Friel and Tsien 1992; Koizumi et al. 1999), store-operated Ca^{2+} influx is observed in neurons from squid olfactory receptors, rat DRG, substantia nigra, and a number of cortical area-derived cultures (Arakawa et al. 2000; Baba et al. 2003; Bouron 2000; Piper and Lucero 1999; Prothero et al. 2000; Tozzi et al. 2003; Usachev and Thayer 1999). For neuroendocrine cells, there is evidence for and against store-operated Ca^{2+} influx in bovine chromaffin cells (Powis et al. 1996; Stauderman and Pruss 1989) as well as proof positive in murine pancreatic β cells (Liu and Gylfe 1997).

Given the presence of voltage-gated Ca^{2+} channels, the role of neuronal store-operated Ca^{2+} influx appears somewhat enigmatic. That said, store-operated channels do initiate secretion in chromaffin cells at membrane potentials too negative for voltage-gated Ca^{2+} channel activation (Fomina and Nowycky 1999). Depleting stores in the hippocampus can also enhance spontaneous transmitter release or induce long-term potentiation (Emptage et al. 2001; Ris et al. 2003). The bag cell neuron afterdischarge is an ~30-min barrage of action potentials that triggers neuropeptide secretion and egg-laying behavior (Conn and Kaczmarek 1989; Kupfermann 1967; Kupfermann and Kandel 1970; Pinsker and Dudek 1977; Rothman et al. 1983). Along with voltage-gated Ca^{2+} channels, store-operated Ca^{2+} influx could promote neuropeptide secretion, particularly late in the afterdischarge when release is maintained despite lowered firing frequency (Loechner et al. 1990; Michel and Wayne 2002). The afterdischarge is also associated with IP$_3$-dependent release of Ca^{2+} from intracellular stores (Fink et al. 1988; Fisher et al. 1994). After the afterdischarge, the bag cells neurons enter a prolonged term of inactivity, known as the refractory period (Kaczmarek and Kauer 1983; Kaczmarek et

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al. 1982). The lack of voltage-gated Ca^{2+} channel opening during the quiescence of the refractory period would hinder Ca^{2+} store repletion; however, this could be circumvented by store-operated Ca^{2+} influx, which would readily replenish Ca^{2+} stores in the absence of activity.

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