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VOLTAGE-GATED Ca^{2+} INFLUX AND MITOCHONDRIAL Ca^{2+} INITIATE SECRETION FROM *APLYSIA* NEUROENDOCRINE CELLS

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Abstract—Neuroendocrine secretion often requires prolonged voltage-gated Ca^{2+} entry; however, the ability of Ca^{2+} from intracellular stores, such as endoplasmic reticulum or mitochondria, to elicit secretion is less clear. We examined this using the bag cell neurons, which trigger ovulation in *Aplysia* by releasing egg-laying hormone (ELH) peptide. Secretion from cultured bag cell neurons was observed as an increase in plasma membrane capacitance following Ca^{2+} influx evoked by a 5-Hz, 1-min train of depolarizing steps under voltage-clamp. The response was similar for step durations of $\geqslant$50 ms, but fell off sharply with shorter stimuli. The capacitance change was attenuated by replacing external Ca^{2+} with Ba^{2+}, blocking Ca^{2+} channels, buffering intracellular Ca^{2+} with EGTA, disrupting synaptic protein recycling, or genetic knock-down of ELH. Regarding intracellular stores, liberating mitochondrial Ca^{2+} with the protonophore, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), brought about an EGTA-sensitive elevation of capacitance. Conversely, no change was observed to Ca^{2+} released from the endoplasmic reticulum or acidic stores. Prior exposure to FCCP lessened the train-induced capacitance increase, suggesting overlap in the pool of releasable vesicles. Employing GTP-γ-S to interfere with endocytosis delayed recovery (presumed membrane retrieval) of the capacitance change following FCCP, but not the train. Finally, secretion was correlated with reproductive behavior, in that neurons isolated from animals engaged in egg-laying presented a greater train-induced capacitance elevation vs quiescent animals. The bag cell neuron capacitance increase is consistent with peptide secretion requiring high Ca^{2+}, either from influx or stores, and may reflect the all-or-none nature of reproduction.

Key words: capacitance, calcium channel, FCCP, CPA, bag cell neurons, peptide release.

INTRODUCTION

Like classical neurotransmission, peptide and neuroendocrine secretion depends on Ca^{2+} influx (Neher, 1998). There is often a delay between voltage-gated Ca^{2+} entry and peptide release, suggesting a Ca^{2+} threshold for secretion (Thomas et al., 1993; Hsu and Jackson, 1996; Branchaw et al., 1998; Sedej et al., 2004). Prolonged periods of action potentials or membrane depolarization are typically more effective than short stimuli at triggering neuropeptide secretion, presumably because this elevates Ca^{2+} over the threshold (Dreifuss et al., 1971; Bicknell and Leng, 1981; Hartzell, 1981; Gainer et al., 1986; Lim et al., 1990; Soldo et al., 2004). In addition, Ca^{2+} from stores can bring intracellular Ca^{2+} closer to the threshold and directly or indirectly influence peptide release (Ammälä et al., 1993; Tse et al., 1997; Shakiryanova et al., 2007; Gilbert et al., 2008). Overall, a threshold may limit hormonally-induced behaviors, particularly high-priority fixed-action patterns that proceed to completion once initiated (Kupfermann and Weiss, 1978).

The bag cell neurons of the marine mollusk, *Aplysia californica*, are neuroendocrine cells that control reproduction through a long-term change in excitability known as the afterdischarge (Kupfermann, 1967; Conn and Kaczmarek, 1989). This burst results from cholinergic and peptidergic inputs, and consists of a fast phase (5-Hz firing for approximately 1 min) and a slow phase (1-Hz firing sustained over 30 min) (Kaczmarek et al., 1982; Brown et al., 1989; White and Magoski, 2012). Intracellular Ca^{2+} rises sharply during the fast phase (Fisher et al., 1994; Michel and Wayne, 2002). *In vivo*, this leads to the neurohaemal release of several peptides, including egg-laying hormone (ELH), which act on central and peripheral targets to evoke egg-laying behavior (Dudek and Tobe, 1978; Chiu et al., 1979; Stuart et al., 1980). Biochemical techniques have revealed Ca^{2+}-dependent peptide secretion from bag

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Abbreviations: ATP, adenosine triphosphate; Ba^{2+}-Cs^{+}-TEA ASW, Ba^{2+}, Cs^{+} tetraethylammonium artificial sea water; Baf, bafilomycin A; Ca^{2+}-Cs^{+}-TEA ASW, Ca^{2+}, Cs^{+} tetraethylammonium artificial sea water; cfASW, Ca^{2+}-free artificial salt water; C_m, membrane capacitance; Cntl, control; CPA, cyclopiazonic acid; DMSO, dimethyl sulfoxide; dsRNA, double-stranded ribonucleic acid; EGTA, ethyleneglycol bis (aminoethylether) tetraacetic acid; ELH, egg-laying hormone; EtOH, ethanol; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazone; GTP, guanosine triphosphate; GTP-γ-S, guanosine-5′-[γ-thio]triphosphate; HEPES, *N*-2-hydroxyethlpiperazine-N′-2-ethanesulphonic acid; I_b, baseline current; IgG, immunogammaglobulin; I_{ss}, steady-state current; MEM, minimum essential medium; nASW, normal artificial salt water; NEM, N-ethylmaleimide; PBS, phosphate-buffered saline; Q_{cf}, correction factor charge; Q_t, total charge; Q_{tc}, charge during transient current; R_a, access resistance; R_m, membrane resistance; SEM, standard error of the mean; tcASW, tissue culture artificial salt water; TEA, tetraethylammonium; TFA, trifluoroacetic acid; TRIS, 2-amino-2-hydroxymethyl-propane-1,3-diol; ΔI, current change to a voltage step; ΔV, step voltage; τ, membrane time constant.

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cell neurons in the intact nervous system following an afterdischarge or high extracellular K^+-mediated depolarization (Arch, 1972; Loechner et al., 1990, 1992; Michel and Wayne, 2002; Hatcher and Sweedler, 2008). The peptides expressed by bag cell neurons are also preserved *in vitro* (Chiu and Stumwasser, 1981), and mass spectrometry has shown ELH release from cultured bag cell neurons subsequent to action potential firing (Hatcher et al., 2005; Jo et al., 2007).

The afterdischarge, neuropeptide secretion, and egg-laying behavior are effectively all-or-none events (Kupfermann, 1967; Ferguson et al., 1989). Yet the relationship between afterdischarge duration and the volume of eggs deposited is less than linear, i.e., even bursts that are shorter than 30 min result in eggs (Dudek et al., 1979). This likely involves the release of Ca^{2+} from intracellular stores promoting ELH secretion subsequent to the afterdischarge (Michel and Wayne, 2002). These circumstances, in combination with the fact that most work on secretion has used the intact cluster, have made it difficult to resolve what duration or pattern of activity is sufficient to cause secretion. Thus, the aim of the present study was to use voltage-clamp and capacitance tracking to characterize how Ca^{2+} entry or release elicits secretion in real time from individual cultured bag cell neurons. Our findings show that only prominent Ca^{2+} elevation, either due to voltage-gated influx or liberation from the mitochondria, initiates secretion. Such conditions fit with a behavior that is fundamental to survival, and may reflect a general principal for the neuroendocrine control of high-threshold actions.

EXPERIMENTAL PROCEDURES

Animals and cell culture

Primary cultures of isolated bag cell neurons were obtained from adult 150–500 g *A. californica* (a hermaphrodite) purchased from Marinus (Long Beach, CA, USA) or Santa Barbara Marine Biologicals (Santa Barbara, CA, USA) and housed in an approximate 300-l aquarium containing continuously circulating, aerated artificial seawater (Instant Ocean; Aquarium Systems; Mentor, OH, USA) at 14–16 °C on 12/12-h light/dark cycle and fed Romaine lettuce 5×/week. All experiments were approved by the Queen's University Animal Care Committee (Protocol No. 100323-Magoski-2012). Following anesthesia by injection of isotonic $MgCl_2$ (around 50% of body weight), the abdominal ganglion was removed and treated for 18 h with neutral protease (13.33 mg/ml; 165859; Roche Diagnostics, Indianapolis, IN, USA) dissolved in tissue culture artificial seawater (tcASW; containing in mM: 460 NaCl, 10.4 KCl, 11 $CaCl_2$, 55 $MgCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), glucose (1 mg/ml), penicillin (100 U/ml), and streptomycin (0.1 mg/ml), pH 7.8 with NaOH). The ganglion was then transferred to fresh tcASW and the two-bag cell neuron clusters dissected from their surrounding connective tissue. Using a fire-polished Pasteur pipette and gentle trituration, neurons were dispersed in simple culture medium (as per tcASW plus minimum essential medium (MEM) vitamins (0.5×; 11120052; Gibco/Invitrogen; Grand Island, NY, USA), MEM non-essential amino acids (0.2×; 11400050; Gibco/Invitrogen), and MEM essential amino acids without L-glutamine (0.2×; 1130051; Gibco/Invitrogen) onto 35 × 10 mm polystyrene tissue culture dishes (430165; Corning, Corning, NY, USA or 353001; Ulti-Dent Scientific, St.-Laurent, QC, Canada) and maintained for 1–3 d in a 14 °C incubator. Experiments were carried out at 22 °C. Salts were from Fisher Scientific (Ottawa, ON, Canada), ICN (Irvine, CA, USA), or Sigma–Aldrich (St. Louis, MO, USA).

Whole-cell voltage-clamp recording

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 external diameter/1.12 internal diameter, borosilicate glass capillaries (TW150F-4; World Precision Instruments, Sarasota, FL, USA) and had a resistance of 1–3 MΩ when filled with various intracellular salines (see below). Pipette junction potentials were nulled immediately before seal formation, while pipette capacitive currents were canceled immediately after break through. To facilitate membrane capacitance tracking (see below), series resistance and whole-cell capacitance were usually not compensated. However, Ca^{2+} currents were occasionally measured separate from capacitance, and in those cases the series resistance (2–5 MΩ) was compensated to 70–80% and monitored throughout the experiment, while the neuronal capacitance was canceled by the whole-cell capacitance compensation. Current was filtered at 1 kHz by the EPC-8 built-in Bessel filter and sampled at 2 kHz using an IBM-compatible personal computer, a Digidata 1300 analog-to-digital converter (Axon Instruments/Molecular Devices; Sunnyvale, CA, USA) and the Clampex acquisition program of pCLAMP 8.1 (Axon Instruments). Clampex was also used to set the holding and command potentials.

In most cases, neurons were held at −80 mV and stimulated with a 5-Hz, 1-min train of square voltage pulses to 0 mV to produce Ca^{2+} influx and secretion (see Results for details). Subtraction of leak from the train-induced Ca^{2+} currents was achieved by subsequently blocking Ca^{2+} channels with 10 mM Ni^{2+}, delivering the train a second time, then subtracting the current in Ni^{2+} from the control current (as per Hung and Magoski, 2007; Tam et al., 2009). In some experiments, neurons were held at −60 mV and Ca^{2+} currents evoked with 200-ms square pulses from −60 mV to +40 mV in 10-mV increments. Leak subtraction from these currents was performed on-line 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.