Acetylcholine-evoked afterdischarge in *Aplysia* bag cell neurons

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Submitted 11 August 2011; accepted in final form 7 February 2012

Acetylcholine-evoked afterdischarge in *Aplysia* bag cell neurons. *J Neurophysiol* 107: 2672–2685, 2012. First published February 8, 2012; doi:10.1152/jn.00745.2011.—A brief synaptic input to the bag cell neurons of *Aplysia* evokes a lengthy afterdischarge and the secretion of peptide hormones that trigger ovulation. The input transmitter is unknown, although prior work has shown that afterdischarges are prevented by strychnine. Because molluscan excitatory cholinergic synapses are blocked by strychnine, we tested the hypothesis that acetylcholine acts on an ionotropic receptor to initiate the afterdischarge. In cultured bag cell neurons, acetylcholine induced a short burst of action potentials followed by either return to near baseline or, like a true afterdischarge, transition to continuous firing. The current underlying the acetylcholine-induced afterdischarge was dose dependent, associated with increased membrane conductance, and sensitive to the nicotinic antagonists hexamethonium, mecamylamine, and α-conotoxin ImI. Whereas nicotine, choline, carbachol, and glycine did not mimic acetylcholine, tetramethylammonium did produce a similar current. Consistent with an ionotropic receptor, the response was not altered by intracellular dialysis with the G protein blocker guanosine 5′-(β-thio)diphosphate. Recording from the intact bag cell neuron cluster showed acetylcholine to evoke prominent depolarization, which often led to extended bursting, but only in the presence of the acetylcholinesterase inhibitor neostigmine. Extracellular recording confirmed that exogenous acetylcholine caused genuine afterdischarges, which, as per those generated synthetically, rendered the cluster refractory to further stimulation. Finally, treatment with a combination of mecamylamine and α-conotoxin ImI blocked synthetically induced afterdischarges in the intact bag cell neuron cluster. Acetylcholine appears to elicit the afterdischarge through an ionotropic receptor. This represents an expedient means for transient stimulation to elicit prolonged firing in the absence of ongoing synaptic input.

**Neuronal communication** typically involves presynaptic neurons evoking postsynaptic action potentials through temporal and spatial summation. However, some neurons display the remarkable property of responding with prolonged firing, long after cessation of the initial stimulus. For these cells, synaptic input results in ionotropic receptor- and/or voltage-gated Ca\(^{2+}\) channel-mediated Ca\(^{2+}\) influx, which acts on nearby channels or causes biochemical change to produce plateau potentials and prolonged depolarization (Andrew and Dudek 1983; Burgoyne 2007; Egorov et al. 2002). Acetylcholine is particularly potent at initiating lengthy bursts through ionotropic receptor-induced depolarization (Elliott et al. 1992; Yamashita and Isa 2003) and lipid metabolism (Tieman et al. 2001), as well as metabotropic channel regulation by second messengers (Haj-Dahmane and Andrade 1996; Yan et al. 2009; Zhang et al. 2011) or tyrosine kinases (Swayne et al. 2009). The present study examines a prolonged response in the bag cell neurons of *Aplysia* following cholinergic ionotropic receptor activation.

The bag cell neurons form two distinct clusters at the base of the pleuroabdominal connectives just rostral to the abdominal ganglion (Coggeshall 1967; Smock and Arch 1977). They are normally silent, but upon brief extracellular stimulation of either the connective or the cerebral ganglion they depolarize and undergo an ~30-min afterdischarge (Ferguson et al. 1989a, 1989b; Kupfermann and Kandel 1970); subsequently, the neurons become refractory to stimulation for ~18 h (Kaczmarek et al. 1982; Magoski et al. 2000; Magoski and Kaczmarek 2005; Zhang et al. 2002). During the afterdischarge, egg-laying hormone (ELH) and other peptides are released into the blood to initiate egg-laying behavior (Arch et al. 1972; Chiu et al. 1979; Sigvardt et al. 1986).

The afterdischarge is a well-studied and profound change in activity and excitability; surprisingly, the input transmitter is unknown, and the only drug found to consistently prevent the afterdischarge is strychnine (Kaczmarek et al. 1978). Although normally considered a glycineic antagonist (Bradley et al. 1953; Curtis et al. 1967), strychnine also blocks cholinergic responses and synapses in *Aplysia* (Faber and Klee 1974; Kehoe 1972b). It is thought that select bag cell neurons receive afferent input from either the pleural or cerebral ganglia (Haskins et al. 1981; Mayeri et al. 1979b). Dye-conjugated microspheres injected into the cluster retrogradely label a small number of neurons in the pleural and cerebral ganglia; conversely, injection into a cerebral ganglion anterogradely stains neurons in each cluster, whereas injection into a pleural ganglion less reliably labels a few ipsilateral cells (Shope et al. 1991). This suggests that a cerebral presynaptic source is more likely.

We now show that acetylcholine is the only candidate neurotransmitter capable of sufficiently depolarizing bag cell neurons to generate afterdischarge-like spiking; moreover, both this response and afterdischarges evoked by cerebral stimulation are blocked by nicotinic antagonists. Our results suggest that acetylcholine in a key input transmitter to the bag cell neurons and demonstrate that transient ionotropic receptor activation can initiate a long-term change in activity fundamental to reproductive behavior.

**Materials and methods**

Animals and cell culture. Adult *Aplysia californica* weighing 150–500 g were obtained from Marinus (Long Beach, CA) and housed in an ~300-liter aquarium containing continuously circulating, aerated sea water (Instant Ocean; Aquarium Systems, Mentor, OH or Sea Chem, Madison, GA) at 15°C on a 12:12-h light-dark cycle and fed romaine lettuce 5 times a week.
For primary cultures of isolated bag cell neurons, animals were anesthetized by an injection of isotonic MgCl₂ (∼50% of body weight), the abdominal ganglion were removed, and animals were treated with neutral protease (13.3 mg/ml; Roche Diagnostics, Indianapolis, IN) dissolved in tissue culture artificial seawater (tcASW; composition in mM: 460 NaCl, 10.4 KCl, 11 CaCl₂, 55 MgCl₂, 15 HEPES, 1 mg/ml glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, pH 7.8 with NaOH) for 18 h at 20–22°C. 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. With the use of a fire-polished Pasteur pipette and gentle trituration, neurons were dispersed in tcASW onto 35 × 10-mm polystyrene tissue culture dishes (430165; Corning, Corning, NY or 353001; Falcon Becton-Dickinson, Franklin Lakes, NJ). Cultures were maintained in 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 and sharp-electrode current-clamp recording from cultured bag cell neurons. Voltage-clamp recordings from cultured bag cell neurons were performed at room temperature (20–22°C) 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, 1.2-mm internal diameter borosilicate glass capillaries (TW150F-4; World Precision Instruments, Sarasota, FL) and had a resistance of 1–2 MΩ when filled with regular intracellular saline (see below). Pipette junction potentials were nullled immediately before seal formation. After seal formation, the pipette capacitive current was canceled, and following breakthrough, the whole cell capacitive current was also canceled, 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 analog-to-digital converter (Molecular Devices, Sunnyvale, CA), and the Clampex acquisition program of pCLAMP (version 8.2; Molecular Devices).

Recordings were made in normal ASW (nASW; composition as per tcASW but lacking glucose and antibiotics) with regular intracellular saline in the pipette [composition in mM: 500 K-aspartate, 70 KCl, 1.25 MgCl₂, 10 HEPES, 11 glucose, 10 glutathione, 5 EGTA, 5 adenosine 5'-triphosphate disodium salt hydrate (ATP; A3377; Sigma-Aldrich), and 0.1 guanosine 5'-triphosphate sodium salt hydrate (GTP; G8877; Sigma-Aldrich); pH 7.3 with KOH]. The free Ca²⁺ concentration was set at 300 nM by adding an appropriate amount of CaCl₂, as calculated using WebMaxC (http://www.stanford.edu/~cspotton/webmaxC.html). A junction potential of 15 mV was calculated for intracellular saline vs. nASW and compensated for by subtraction off-line.

Current-clamp recordings were made from cultured bag cell neurons in nASW by using an AxoClamp 2B (Axon Instruments/Molecular Devices) amplifier and the sharp-electrode, bridge-balanced method. Microelectrodes were pulled from 1.2-mm external, 0.9-mm internal diameter borosilicate glass capillaries (TW120F-4; World Precision Instruments) and had a resistance of 5–20 MΩ when filled with 2 M K-acetate plus 10 mM HEPES and 100 mM KCl (pH 7.3 with KOH). Current was delivered with either Clampex or a Grass S88 stimulator (Astro-Med, Longueuil, QC, Canada). Voltage was filtered at 3 kHz using the Axoclamp Bessel filter and sampled at 2 kHz as appropriate.

Ensemble extracellular and single-neuron sharp-electrode current-clamp recording from the intact bag cell neuron cluster. For extracellular recording, the abdominal ganglion with or without the attached central ring ganglia was maintained in a 14°C nASW-filled chamber. A wide-bore, fire-polished glass suction recording electrode (containing nASW) was placed over one of the two bag cell neuron clusters, while a similar stimulating electrode was placed either at the rostral end of the pleuroabdominal connective corresponding to the recorded cluster or over the rostromedial area of the right cerebral ganglion. In most instances when the central ring was included, it was isolated from the abdominal ganglion by means of a custom-made chamber with a thin plastic barrier. The pleuroabdominal connectives passed through two small slits in the barrier and were covered with Vaseline for complete isolation (see RESULTS for details). Stimulating current pulses were delivered with a Grass S88 stimulator and isolation unit while voltage was monitored using a Warner DP-301 differential amplifier (Warner Instruments, Hamden, CT). Voltage was high-pass filtered at 0.1 Hz and low-pass filtered at 1 kHz using the DP-301 filters and acquired at a sampling rate of 2 kHz using Axoscope (version 9.0; Molecular Devices).

For intracellular recording from single bag cell neurons in the intact cluster, the sharp-electrode, bridge-balanced method was again employed, but in this case with the use of a Neuroprobe 1600 amplifier (A-M Systems, Sequim, WA). To facilitate sharp-electrode impalement, ganglia were treated with 0.5 mg/ml elastase (E1250; Sigma-Aldrich) and 1 mg/ml collagenase/dispase (10269638001; Roche) for 2 h (Fisher et al. 1994; Kehoe 1972c), and then one of the clusters (usually the left) was desheathed using fine forceps. Voltage was filtered at 1 kHz using the Neuroprobe filter and acquired with Axoscope at 2 kHz. Extra- and intracellular recordings were sometimes carried out simultaneously by recording extracellularly from the cluster that was ipsilateral to the stimulated right cerebral ganglion while recording intracellularly from a neuron in the contralateral cluster.

Drug application and reagents. For cultured bag cell neurons, the culture dish served as the bath, with transmitters and drugs being applied directly using a single-cell microperfusion system at ∼1 μl/min. The perfusion system consisted of a micromanipulator-controlled square-barreled glass pipette (∼500-μm bore) positioned 300–500 μm from the soma and connected by a stopcock manifold to a series of gravity-driven reservoirs. This provided a constant flow of control extracellular saline over the neuron, which was switched to agonist-containing saline for a specific amount of time by activating the appropriate stopcock. Additional experiments, particularly those involving acetylcholine agonists, saw the blocker introduced directly into the bath by pipetting a small volume of concentrated stock solution prior to pressure ejection of acetylcholine from an unpolished patch pipette (1- to 2-μm bore) for 2 s at 75–150 kPa using a PMI-100 pressure microinjector (Dagan, Minneapolis, MN). As was previously undertaken with bag cell neurons by Fisher et al. (1993), no perfusion was employed during the pressure application protocol; however, the pipette was removed from the bath immediately after each ejection to minimize leakage and possible desensitization.

All drugs were made up as stock solutions in water and frozen at −20°C and then diluted down to a working concentration daily as needed: α-conotoxin Im1 (3119; Tocris Bioscience, Bristol, UK); acetylcholine chloride (A6625; Sigma-Aldrich); carbacbol (212385; Calbiochem); choline chloride (C1879; Sigma-Aldrich); guanosine 5'- (β-thio)diphosphate trillithium salt (GDPS) (G7637; Sigma-Aldrich); glycine (G7126; Sigma-Aldrich); hexamethonium chloride (H2138; Sigma-Aldrich); mecamylmine hydrochloride (M9020; Sigma-Aldrich); methyllycaconitine citrate salt hydrate (M168; Sigma-Aldrich); neostigmine bromide (N2001; Sigma-Aldrich); nicotine (N0257; Sigma-Aldrich); oxotremorine sesquifumarate (O9126; Sigma-Aldrich); Phe-Met-Arg-Phe amide (FMRFamide; F4898; Sigma-Aldrich); serotonin (H9523; Sigma-Aldrich); strychnine (S0532; Sigma-Aldrich); and tetramethylammonium chloride (TMA; T19526; Sigma-Aldrich).

Analysis. The Clampfit analysis program of pCLAMP was used to determine the amplitude and time course of changes to membrane potential or holding current evoked by candidate neurotransmitters and drugs under current- or voltage-clamp. After at least 1 min of baseline, two cursors were placed immediately prior to the current or voltage change; while an additional two cursors were similarly positioned at the peak of the response (see RESULTS for details). Clampfit...
then calculated the average current or voltage between the paired cursors. The maximal amplitude of the response was taken as the difference between these average baseline and peak values. Current was normalized to cell size by dividing by the whole cell capacitance (as determined by the EPC-8 slow capacitance compensation circuitry). For display, most current and voltage traces were filtered off-line between 20 and 80 Hz using Clampfit. The slow nature of the responses ensured that this second filtering brought about no change in amplitude or kinetics. Conductance was derived using Ohm’s law \( G = I/V \) from the current during a 200-ms step from \(-60 \) to \(-70 \) mV. In cases where acetylcholine was applied twice, the subsequent application occurred after an \(~10\)-min interval, with the peak current of the second response expressed as a percentage of the first response.

Afterdischarge duration was quantitated as the time from the initial extracellular spike after cessation of the stimulus. In some cases, the response went beyond 30 min and was truncated at that point. For display, extracellular voltage was filtered off-line to 80 Hz using Clampfit.

Data are means \( \pm \) SE. Statistical analysis was performed using Instat (version 3; GraphPad Software, San Diego, CA). The Kolmogorov-Smirnov method was used to test data sets for normality. To test whether the mean differed between two groups, either Student’s unpaired \( t \)-test (for normally distributed data) with the Welch correction as necessary (for unequal standard deviations) or the Mann-Whitney \( U \)-test (for not normally distributed data) was used. For three or more means, multiple comparisons involved an analysis of variance (ANOVA) followed by Dunn’s multiple comparison post hoc test. Fisher’s exact test, which examines the association between two variables, was used to test differences in frequency. The level of significance of the one- or two-tailed \( P \) value was set at \(<0.05\).

RESULTS

Prior work shows that most Aplysia neurons tested are acetylcholine responsive, with the vast majority in the abdominal ganglion presenting hyperpolarization and a minority showing either depolarization or a combined depolarizing-hyperpolarizing response (Frazier et al. 1967; Tauc and Gerfenfeld 1961). As such, at least four cholinergic receptor types are believed to exist: two distinctive \( Cl^- \) conductances, one rapidly desensitizing and the other slowly desensitizing, mediate the hyperpolarizing responses (Gardner and Kandel 1977; Kehoe 1972c; Kehoe and McIntosh 1998); the depolarizing response is due to a nonselective cation conductance (Ascher et al. 1978a; Marty et al. 1976); and a fourth acetylcholine-induced response involves a \( K^+ \) protein-dependent \( K^+ \) conductance (Kehoe 1994). Some Aplysia neurons contain only one receptor type, yet others, such as those in the medial pleural ganglion, present both depolarizing and hyperpolarizing responses (Kehoe 1972b). The response of bag cell neurons to acetylcholine is not well characterized, with only one previous study noting modest depolarization (Bodmer and Levitan 1984).

Acetylcholine depolarizes cultured bag cell neurons. To satisfy the role of an input transmitter triggering the afterdischarge, acetylcholine should depolarize and evoke action potentials in bag cell neurons. Microperfusion of 1 mM acetylcholine dissolved in nASW near the soma of a cultured bag cell neuron (see MATERIALS AND METHODS for details) induced a substantial depolarization under sharp-electrode current clamp at \(-60 \) mV \((n = 20)\). With continued exposure of agonist, the peak depolarization lasted a few seconds before returning to baseline within 3–5 min. In some cases, the response did not return to baseline but reached a new, depolarized steady state. Acetylcholine delivered in this manner depolarized 2 of the 20 bag cell neurons sufficiently to induce spiking (not shown). To speed the rate of the depolarization, 1 mM acetylcholine was pressure applied for 1–2 s near the soma \((n = 13)\) (see MATERIALS AND METHODS for details) and produced a more robust and rapid response that consistently resulted in a brief burst of action potentials in 10 of the 13 neurons tested (Fig. 1A). Although the same concentration of acetylcholine was used for the initial microperfusion and pressure application experiments, it is likely that the latter delivered a more rapid change in agonist concentration, allowing the neuron to reach threshold more consistently before desensitization occurred (see below). When the data sets for the acetylcholine-induced depolarization evoked by either microperfusion or pressure ejection were merged, the average was significantly different from zero at 36.8 \( \pm \) 2.73 mV \((n = 33)\) (Fig. 1B). Occasionally, this depolarization led to afterdischarge-like spiking that continued long after the stimulus \((n = 5)\) (Fig. 1C). In these cases, the depolarization followed a similar initial time course; however, instead of returning to \(-60 \) mV, the neuron reached a depolarized steady state of approximately \(-40 \) mV and proceeded to fire action potentials. This is similar to potentials recorded intracellularly from neurons in the cluster during an afterdischarge caused by presynaptic stimulation (Fisher et al. 1993; Kauer and Kaczmarek 1985; Mayeri et al. 1979b), as well as from cultured neurons responding to a brief train of action potentials (Hung and Magoski 2007) or application of cAMP (Kaczmarek and Strumwasser 1981).

A concentration-dependent, acetylcholine-induced inward current. To further explore the acetylcholine-induced response, we characterized the properties of the underlying current in cultured bag cell neurons. Initial testing showed that application of acetylcholine by microperfusion, for as short as 10 s, caused marked desensitization to subsequent applications, even following a 20-min wash. This may explain the difficulty we encountered in evoking spiking with this method (see above). Therefore, acetylcholine was applied only once per neuron by microperfusion over the soma under whole cell voltage clamp at \(-60 \) mV using a \( K^+ \)-based intracellular saline. Figure 2A shows example traces from four concentrations \((10, 100, 300, \) and \(1,000 \) \(\mu M\)) in separate neurons, revealing a relatively slow-onset current at lower concentrations, becoming faster at higher doses. Return to baseline typically took 3–5 min in the continued presence of agonist. Delivering concentrations ranging from 1 \(\mu M\) to 10 mM generated a dose-response curve with a Hill coefficient of 0.7, indicating either a lack of cooperativity or potential negative cooperativity, and an EC\(_{50}\) of 267 \(\mu M\) (Fig. 2B).

Ascher et al. (1978a) showed that the depolarizing response to acetylcholine in right pleural ganglion neurons involved an increased cation conductance. To test whether the bag cell neuron current was due to channel opening, conductance was calculated using a 200-ms step from \(-60 \) to \(-70 \) mV (Fig. 2C, bottom). Three of these \(-10\)-mV steps were delivered to each neuron: two before the acetylcholine perfusion, separated by 1–2 min, and one at the peak of the acetylcholine-induced response. To account for leak and achieve a baseline conductance change over time, the first control current evoked by the step was subtracted from the second current (Fig. 2C, top). The second control current was then subtracted from the current produced by the step during the acetylcholine
response (Fig. 2C, middle). The increase in conductance change between the peak acetylcholine response and the prior control period was clear and readily met the level of significance (n = 9) (Fig. 2D).

Because nicotinic vs. muscarinic receptor classification is not clearly delineated in molluscs, it was necessary to see whether oxotremorine, a muscarinic agonist shown to be effective in Aplysia (Dembrow et al. 2004), would mimic the acetylcholine-induced current. However, bath application of 20 μM oxotremorine failed to produce an appreciable current at −60 mV (n = 4) (Fig. 2, E and F). Furthermore, since strychnine, the only agent known to block the afterdischarge (Kaczmarek et al. 1978), is typically thought of as a glycinergic blocker (Bradley et al. 1953; Curtis et al. 1967), we also tested Gly, the only agent known to block the afterdischarge (Kaczmarek et al. 1978), is typically thought of as a glycinergic blocker (Bradley et al. 1953; Curtis et al. 1967), we also tested Gly. The evidence thus far suggests that the bag cell neuron acetylcholine current is ionotropic; however, if acetylcholine acts in a metabotropic manner, it would be G protein coupled and blocked by GDPβS (a nonhydrolyzable form of GDP) (Eckstein et al. 1979). Dialysis of cultured bag cell neurons for 30 min with normal internal solution plus 10 mM GDPβS (replacing the 0.1 mM GTP) did not alter the current to pressure-applied acetylcholine (1 mM) at −60 mV (n = 5) (Fig. 3A, right), compared with parallel controls dialyzed with internal solution containing GTP (n = 7) (Fig. 3A, left). Summary data indicate a slight but not significant increase in the acetylcholine-induced current with GDPβS in the pipette (Fig. 3B). As a positive control, the outward current at −40 mV produced by pressure-applied FMRFamide (500 μM), a known metabotropic agonist in molluscs (Brezina 1988; Fisher et al. 1993; Piomelli et al. 1987a, 1987b), was blocked by GDPβS (Fig. 3, C and D). The summary data of FMRFamide following GDPβS dialysis in Fig. 3D include data from neurons never exposed to acetylcholine (n = 4/8) as well as 10 min after application of acetylcholine (n = 4/8), which were not significantly different from each other (data not shown, 2-tailed Mann-Whitney) and thus were pooled.

Acetylcholine response is sensitive to specific cholinergic blockers. Most cholinergic currents of Aplysia neurons are blocked by traditional nicotinic antagonists; in particular, the...
inward current is blocked in a voltage-dependent manner by hexamethonium or D-tubocurarine (Ascher et al. 1978b; Kehoe 1972b) and in a voltage-independent manner by α-conotoxin ImI (Kehoe and McIntosh 1998). Because D-tubocurarine also blocks dopamine receptors in molluscan neurons (Ascher 1972; Berry and Cottrell 1975), we chose to examine the effects of the general antagonists hexamethonium (Paton and Zaimis 1948; Tauc and Gerschenfeld 1961), mecamylamine (Ascher et al. 1979; Stone et al. 1956; Ueki et al. 1961), methyllycaconitine (MLA), which at low doses preferentially blocks α7 nicotinic receptors (Alkondon et al. 1992; Mogg et al. 2002; Ward et al. 1990), and α-conotoxin ImI (Johnson et al. 1994; McIntosh et al. 1994). Choline, which can preferentially activate α7 nicotinic receptors (Alkondon et al. 1997; Papke et al. 1996), was also tested for agonist-induced desensitization.

A pair of 2-s acetylcholine (1 mM) pressure applications, separated by a minimum of 10 min, were used and expressed as a percentage of the second current vs. the first. Antagonists were introduced into the bath after the first acetylcholine application, and the relative effectiveness was determined by calculating the percent remaining current evoked by the second application. Under control conditions, without the addition of any antagonist to the bath, the second application of acetylcholine produced a peak current of 54.7 ± 3.5% of the first (n = 26) (Fig. 4, A and G). Bath application of 500 μM hexamethonium (n = 6) (Fig. 4, B and G), 100 μM mecamylamine (n = 12) (Fig. 4, C and G), or 1 μM α-conotoxin ImI (n = 6) (Fig. 4, D and G) after the initial current had returned to baseline blocked much of the second acetylcholine-induced current. The postmecamylamine current was rapid, whereas the posthexamethonium or post-α-conotoxin ImI currents were relatively slow. Delivering both mecamylamine and α-conotoxin ImI together virtually eliminated the current evoked by the second acetylcholine application (n = 5) (Fig. 4, E and G). For either 1 μM MLA or 10 mM choline, only 1 of the 7 neurons showed a measurable block; therefore, the average of total remaining current in both cases was not significantly.

Fig. 2. Current responses in cultured bag cell neurons to the application of ACh and related agonists. A: whole cell voltage-clamp recordings in nASW with K+-based internal solution. Current induced by continuous microperfusion of 10 μM, 100 μM, 300 μM, or 1 mM Ach at a holding potential of −60 mV. B: the dose-response curve for Ach reveals an EC50 of 267 μM with little cooperativity, based on a Hill coefficient of 0.70 (n = 2 for 1 μM, 5 for 3 μM, 3 for 10 μM, 6 for 30 μM, 16 for 100 μM, 6 for 300 μM, 9 for 1 mM, 8 for 3 mM, and 7 for 10 mM). C: subtracted current traces taken 1 min apart under voltage clamp during a 10-mV hyperpolarizing step (bottom). Before the addition of Ach, essentially no conductance change occurs (top); however, subtraction currents taken at the peak of the ACh response demonstrate a large increase in conductance (middle). D: percent change in conductance (ΔG), from 19.4 ± 7.9% between consecutive steps in control conditions to 2,935 ± 829% between steps in control and Ach, was significantly different (*P < 0.01, 2-tailed paired Student’s t-test). E: example current traces show that microperfusion of oxotremorine (oxo), a muscarinic agonist, and glycine, a member of the cys-loop ligand-gated ion channel family, of which the nicotinic receptor is also a member, do not induce a current. F: summary graph of different agonists used. Only tetramethylammonium (TMA) produced a current (~4.5 ± 0.8 pA/pF) similarly to Ach (~5.8 ± 0.7 pA/pF). The other agonists, including nicotine (0.12 ± 0.01 pA/pF), were all significantly different (P < 0.01, 1-way ANOVA, Dunnett’s multiple comparisons test). Null currents were included in the summary of serotonin (5-HT)-induced responses for a mean of 0.21 ± 0.13 pA/pF. HP, holding potential.
different from control (n = 7 and 7) (Fig. 4G). Considering that strychnine can block the afterdischarge (Kaczmarek et al. 1978) as well as cholinergic synaptic transmission in *Aplysia* central ring neurons (Faber and Klee 1974; Kehoe 1972a), we also tested its effects on cultured bag cell neurons. Compared with control, 500 μM strychnine significantly blocked the second acetylcholine-induced current (n = 12), although not to the same extent as mecamylamine or α-conotoxin ImI (Fig. 4, F and G).

**Block of the acetylcholine response shows differential voltage dependence.** During the afterdischarge, the bag cell neurons are typically depolarized from a resting potential of −60 mV to spiking potential of between −40 and −30 mV (Kaczmarek et al. 1982; Mayeri et al. 1979a, 1979b). Because of the voltage-dependent block by many of the nicotinic antagonists used here (Ascher et al. 1978b; Kehoe and McIntosh 1998), we examined whether hexamethonium, mecamylamine, and α-conotoxin ImI were still able to inhibit the cholinergic current at depolarized potentials. While holding at −30 mV, a second pressure application of 1 mM acetylcholine induced a current 68.0 ± 4.9% of the first (n = 8) (Fig. 5A), which failed to reach the level of significance compared with the run-down at −60 mV (Fig. 5F). At −30 mV, 500 μM hexamethonium was essentially the same as control, with the current at 56.7 ± 5.5% of the initial value (n = 6) (Fig. 5B), and significantly different from the 16.6 ± 5.2% remaining current at −60 mV (Fig. 5F). Similarly, 100 μM mecamylamine resulted in a 22.5 ± 8.8% decrease of the initial current at −30 mV (n = 6) (Fig. 5C), which again was significantly less than the 7.7 ± 3.3% remaining current at −60 mV (Fig. 5F). Clearly, hexamethonium and, to a lesser extent, mecamylamine are not effective antagonists of the cholinergic current at depolarized potentials. α-Conotoxin ImI (1 μM), on the other hand, was equally potent at blocking the current, with 14.0 ± 3.1% and 17.1 ± 8.5% remaining current at −60 and −30 mV (n = 8), respectively (Fig. 5, D and F). Moreover, as was the case at −60 mV, a combination of mecamylamine and α-conotoxin ImI completely blocked the second current at −30 mV (n = 6) (Fig. 5, E and F).

**Acetylcholine depolarizes bag cell neurons in the intact cluster.** Having established that bag cell neurons respond to acetylcholine with an ionotropic receptor, we next explored the response of neurons in the intact bag cell neuron cluster to acetylcholine. The bag cell neuron cluster contains a high concentration of acetylcholinesterase, similar to levels observed in neuropil of the abdominal ganglion (Giller and Schwartz 1971). Therefore, most experiments employed the acetylcholinesterase inhibitor neostigmine, which has been previously shown to counteract the enzyme while not directly affecting cholinergic currents in *Aplysia* neurons (Ascher et al. 1978b; Bhattacharya and Feldberg 1958; Carpenter et al. 1976; Slater et al. 1986). In addition, nASW was used in favor of high-divalent seawater, given that the latter greatly reduces the likelihood of afterdischarge generation (Kaczmarek et al. 1982).

For intracellular recording, single bag cell neurons within the cluster were sharp-electrode current-clamped to −60 mV and acetylcholine was bath-applied (see MATERIALS AND METHODS for details). In four neurons tested without neostigmine pretreatment, acetylcholine (1 mM) produced an average depolarization of 6.1 ± 2.2 mV (Fig. 6, A and D). A 20-min pretreatment with 2 μM neostigmine increased the cholinergic depolarization to 24.8 ± 2.6 mV (n = 17 neurons from separate clusters in different abdominal ganglia) (Fig. 6, B and D). In 7 of the 17 bag cell neurons that depolarized, acetylcholine induced continuous spiking similar to that of an afterdischarge (Fig. 6C). However, it was not possible to accurately measure the duration of acetylcholine-induced afterdischarges using intracellular recording because of difficulty in maintaining recordings of consistent duration. Acetylcholine likely activated muscles within the pleuroabdominal connective (Coggeshall 1967) or neurons within the abdominal ganglia that project to intrinsic muscles (Alevizos et al. 1991), which served to dislodge the recording electrode. Thus we also employed a dual recording of the contralateral cluster using an extracellular suction electrode (see MATERIALS AND METHODS for details). In 4 extracellularly recorded clusters, acetylcholine induced an afterdischarge of 6.9 ± 1.2 min in duration (Fig. 6, E and F).

**Prior acetylcholine exposure changes the response of the bag cell neuron cluster.** If acetylcholine were an input transmitter for the afterdischarge, it would be expected that clusters stimulated by acetylcholine would more likely be refractory. Typically, bag cell neurons can be induced to fire an afterdischarge through extracellular stimulation of the pleuroabdominal connective with a 5-Hz, 10- to 30-s stimulus protocol...
However, some bag cell neuron processes can travel up the connective to reach the pleural ganglion (Haskins et al. 1981); thus, to avoid as much as possible the antidromic stimulation of bag cell neuron processes, we chose to stimulate the right cerebral ganglion near the region of the F cluster as per Ferguson et al. (1989b). The stimulation protocol involved increasing the extracellular voltage from 10 to 25 V until the initiation of extracellular spikes. Stimulation was terminated 5 s after the first appearance of extracellular spikes or at 30 s. If no afterdischarge was initiated, a second 30-s protocol was delivered after a 10-min rest period.

Stimulation of 25 separate bag cell neuron clusters consistently generated an afterdischarge with an average duration of 14.6 ± 3.0 min (Fig. 7A, left). However, in 11 clusters previously exposed to 1 mM acetylcholine in 2 μM neostigmine, stimulation failed to evoke an afterdischarge in 9 cases (Fig. 7B), whereas the remaining 2 clusters presented durations of 7 and 17 min, respectively. This decreased frequency of afterdischarge generation following prior acetylcholine exposure was significant (Fig. 7A, right). Conversely, it was expected that clusters previously stimulated to the point of refractoriness would also fail to respond to acetylcholine. Figure 7C (left) shows an example of an intracellular recording from an intact bag cell neuron, in the presence of neostigmine, and the afterdischarge response to synaptic input. In four separate clusters, such previous stimulation rendered 1 mM acetylcholine ineffective at generating an afterdischarge (Fig. 7C, right). Two clusters did not respond at all to acetylcholine, whereas one cluster hyperpolarized and another depolarized slightly but not sufficiently to induce action potentials.

Mecamylamine and α-conotoxin ImI block the afterdischarge. Because the addition of mecamylamine and α-conotoxin ImI could block the cholinergic current in cultured bag cell neurons at both resting and depolarized membrane potentials, it was important to see whether these antagonists also blocked the afterdischarge evoked by presynaptic stimulation. One cluster was recorded per animal. In control conditions, without the addition of antagonists, cerebral stimulation induced an afterdischarge of 21.0 ± 3.9 min in duration (n = 8) (Fig. 8A and C, left), which became refractory to subsequent stimulation. Pretreatment with both 100 μM mecamylamine and 1 μM hexamethonium (500 μM) potently blocks the second ACh-induced current, reducing it to 16.6 ± 5.2, 7.7 ± 3.3, or 14 ± 3.1% of the initial value, respectively. E: adding both mecamylamine and α-conotoxin ImI simultaneously almost completely eliminates the second response to ACh (1.0 ± 0.4% of the initial current). F: in a separate neuron, bath application of strychnine (500 μM) after the first ACh response further reduces the current evoked by the second ACh application to 23.9 ± 5.2% of the initial value. G: summary data showing the percentage of remaining current between the second and first ACh applications. Compared with control, there is significantly less residual current in the presence of hexamethonium, mecamylamine, α-conotoxin ImI, mecamylamine and α-conotoxin ImI together, or strychnine, but not with methyllycaconitine (MLA), an α7 nicotinic receptor-selective antagonist, or choline, an α7 nicotinic receptor desensitizing agonist (*P < 0.001, 1-way ANOVA, Dunn’s multiple comparisons test). hex, Hexamethonium; mec, mecamylamine; ImI, α-conotoxin ImI.
α-conotoxin ImI completely blocked any afterdischarge due to stimulation in 5 of 7 clusters (Fig. 8, B, left, and C, left). The other two clusters responded with normal afterdischarges of 18 and 30 min, respectively. The latter indicates the possibility that multiple factors may initiate an afterdischarge (see Discussion). For the 5 clusters blocked by the antagonists, subsequent stimulation after a 30-min wash restored the ability to generate an afterdischarge in 3 cases, with an average duration of 6.7 ± 0.5 min (Fig. 8, B, right, and C, left). The presence of mecamylamine and α-conotoxin ImI produced a significant decrease in the frequency of afterdischarge generation induced by cerebral stimulation compared with control (Fig. 8C, right).

**DISCUSSION**

We present evidence for acetylcholine being an input transmitter to the bag cell neurons. First, acetylcholine is the only tested transmitter capable of sufficiently depolarizing bag cell neurons, both in culture and within the intact cluster, to action potential threshold. Some cultured neurons display regenerative firing long after cessation of agonist application. In the intact cluster, the addition of the acetylcholinesterase inhibitor neostigmine enables acetylcholine to induce an afterdischarge. Second, a combination of the nicotinic antagonists mecamylamine and α-conotoxin ImI block both the acetylcholine-induced current in cultured neurons and afterdischarges in the intact cluster evoked by cerebral ganglion stimulation. Third, refractory clusters are much less likely to respond to acetylcholine, whereas resting clusters exposed to acetylcholine become refractory to synaptic stimulation. Thus, along with a number of other factors (see below), acetylcholine has the potential to initiate the afterdischarge in vivo.

**Strictly depolarizing response of acetylcholine in cultured bag cell neurons.** Based on an increase in conductance at peak current and a lack of attenuation following GDPβS dialysis, the bag cell neuron acetylcholine response is consistent with an inward current through opening of an ionotropic receptor. GDPβS is well established as preventing metabotropic receptor coupling in molluscan neurons (Brezina 1988; Kehoe 1994; Lemos and Levitan 1984; Magoski et al. 1995; Tam et al. 2011). Furthermore, the metabotropic agonist oxotremorine did not elicit a current, and in no instance did acetylcholine induce hyperpolarization or outward current in cultured bag cell neurons. Within the intact ganglia, acetylcholine occasionally caused hyperpolarization; however, since it was bath applied in nASW, extrasynaptic events may have contributed. The acetylcholine current we observe mimics the current mediated by a nonselective cation channel-type ionotropic receptor in *Aplysia* right pleural ganglion neurons (Ascher et al. 1978a, 1978b; Kehoe and McIntosh 1998; Marty et al. 1976). The acetylcholine current we observe mimics the current mediated by a nonselective cation channel-type ionotropic receptor in *Aplysia* right pleural ganglion neurons (Ascher et al. 1978a, 1978b; Kehoe and McIntosh 1998; Marty et al. 1976). Both responses show voltage-dependent block by hexamethonium or mecamylamine and voltage-independent block by strychnine or α-conotoxin ImI, and they are recapitulated by TMA but not nicotine. As per Ascher et al. (1978b) and Kehoe and McIntosh (1998), we find hexamethonium block to be strongly voltage dependent (essentially not effective at −30 mV) and mecamylamine block somewhat less influenced by...
depolarization. However, unlike other reports, the bag cell neuron current presents long-lasting desensitization and is insensitive to carbachol. The latter was surprising but not unprecedented, since carbachol cannot reproduce the excitatory effect of acetylcholine on Aplysia gill (Weiss et al. 1984) or parapodial muscle (Laurienti and Blankenship 1999).

Similarities to molluscan and mammalian nicotinic receptors. The one cloned molluscan cation-selective acetylcholine receptor is from Lymnaea (LnAchRA), and although it is blocked by mecamylamine and α-conotoxin ImI but not MLA, it differs from the bag cell neuron current by showing nicotine and choline sensitivity, as well as faster kinetics and little desensitization to repeated applications (van Nierop et al. 2005). The bag cell neuron cholinergic current is probably not mediated by an α7 nicotinic receptor, because the Aplysia α7-like conductance is Cl− selective, reproduced by nicotine, and blocked by MLA (Kehoe and McIntosh 1998). The most striking difference between the bag cell neuron current and vertebrate nicotinic receptors is a lack of mimicry by nicotine. Although nicotine can elicit an inward current, we find it differs from the acetylcholine-gated channel in reversal potential, cooperativity, Ca2+ sensitivity, and antagonist profile (unpublished observations).

Presynaptic role for acetylcholine in afterdischarge generation. Either the afterdischarge is initiated by a cholinergic afferent input (likely from the cerebral ganglia), or the bag cells themselves are cholinergic and secrete acetylcholine to maintain the afterdischarge. Previous evidence indicates synaptic input is more likely, i.e., the bag cell neurons are not labeled by anti-acetylcholine immunohistochemistry (Soinila and Mpitsos 1991). Also, the levels of acetylcholinesterase, which would breakdown acetylcholine released from any input, are comparable between the bag cell neuron cluster and the synaptic neuropil of the abdominal ganglion (where cholinergic transmission is expected to be common) (Giller and Schwartz 1971). Moreover, if the bag cell neurons were cholinergic, a significant amount of choline acetyltransferase would be required to synthesize acetylcholine, yet the cluster contains only a low level of this enzyme (Giller and Schwartz 1968; McCaman and Dewhurst 1970). It is likely that processes traveling through or innervating the cluster give rise to this small signal. Further evidence for potential cholinergic input is the presence of small, clear vesicles near select bag cell neuron axon bundles; these are in contrast to most bag cell neuron neurites, which contain moderately dense core granules and innervate the connective tissue sheath (Chiu and Strumwasser 1981; Haskins et al. 1981).

Strychnine can block excitatory postsynaptic potentials from a presumed cholinergic input to anterior pleural cells (Kehoe 1972c), as well as the afterdischarge in bag cell neurons (Kaczmarek et al. 1978). Tubocurarine or hexamethonium was unable to block the afterdischarge in intact clusters (Kaczmarek et al. 1978). However, tubocurarine is known to inhibit receptors for other transmitters (Ascher 1972; Berry and Cottrell 1975), and such lack of specificity may explain why it was unable to prevent the afterdischarge. In addition, both ourselves and others (Kehoe and McIntosh 1998) found hexamethonium to be a poor blocker at −30 mV. Because the after-

Fig. 6. Intracellular and extracellular responses of bag cell neurons to Ach within the intact cluster from the abdominal ganglion. A: under sharp-electrode current clamp, an individual bag cell neuron is depolarized to a small extent by bath-applied Ach (1 mM) but not sufficiently to induce action potentials. B: with a 30-min pretreatment in the acetylcholinesterase inhibitor neostigmine (2 μM), the Ach-induced response is increased dramatically. C: in 7 of the 17 neurons within individual bag cell neuron clusters from different animals, the Ach-induced depolarization is sufficient to cause maintained action potential firing. D: the addition of neostigmine to the bath significantly increases the Ach response of individual bag cell neurons within the intact cluster 4-fold (from 6.1 ± 2.2 to 24.8 ± 2.6 mV) (*P < 0.01, 2-tailed Mann-Whitney U-test). E: −1 min after bath application of Ach, the bag cell neuron cluster underwent an afterdischarge characterized by the presence of extracellular spikes. F: summary data showing an average Ach-induced afterdischarge of 6.9 ± 1.2 min.
discharge is associated with depolarization to −40 or even −30 mV (Kaczmarek et al. 1982; Mayeri et al. 1979a, 1979b), hexamethonium could have been rendered ineffective. We have shown that the afterdischarge is blocked by a combination of the specific nicotinic antagonists mecamylamine and α-conotoxin IImL. This cocktail also eliminates the acetylcholine-induced current in cultured bag cell neurons at both resting and depolarized membrane potentials. That stated, we cannot rule out the possibility that the antagonists may also act on the receptors for some unknown chemical input.

Possible cholinergic synaptic input to the bag cell neurons. Surprisingly little is known about the synaptic input to bag cell neurons; however, the homologous caudodorsal cells in <i>Lymnaea</i> receive cholinergic afferents (Ter Maat et al. 1983). Most bag cell neuron axons project to the vascularized sheath and rarely course more than 1 cm rostrally along the pleuroabdominal connective (Haskins et al. 1981). Nevertheless, some axons run through the connective core to the pleural or cerebral ganglia, and retrograde labeling of bag cell inputs reveals neurons in the pleural and cerebral ganglia (Shope et al. 1991). Furthermore, cobalt backfilling of the connective stains a number of neurons in the cerebral ganglion at a site where focal extracellular stimulation induces afterdischarges (Ferguson et al. 1989b).

The cerebral F-cluster neurons have been implicated in bag cell neuron function (Ferguson et al. 1989b; Rubakhin et al. 1999). Neurons that stain for acetylcholine are observed near the F cluster (Soinila and Mptisos 1991), but their actual location has not been accurately described, so it is possible that we directly stimulated these cells in addition to the F-cluster neurons. The F cluster is bordered by the C cluster, which contains ectopic bag cell neurons, small populations of neurons in both the cerebral and pleural ganglia that produce ELH and other bag cell neuron peptides (Chiu and Strumwasser 1981, 1984). Even if focal stimulation spread to ectopic bag cell neurons in the cerebral C cluster, the latter send their processes into the neuropil and not down the connective, and they are ineffective at initiating afterdischarges on their own (Chiu and Strumwasser 1984; Painter et al. 1989).

**Acetylcholine-induced long-term change in activity.** Acetylcholine generates plateau potentials in cortical and hippocampal neurons by coupling muscarinic receptors to nonselective cation channels (Egorov et al. 2002; Tahvildari et al. 2008; Tai et al. 2010; Zhang and Seguela 2010; Zhang et al. 2011). Nevertheless, nicotinic receptors in substantia nigra neurons produce moderate bursting in a Ca²⁺-dependent manner (Yamashita and Isa 2003). Acetylcholine evokes plateau potentials and bursting in <i>Lymnaea</i> N1 and N2 feeding neurons through a presumed ionotropic receptor (Brierley et al. 1997; Elliott and Kemenes 1992; Straub et al. 2002). Also, cerebral cholinergic neurons modulate the <i>Aplysia</i> feeding central pattern generator (Dembrow et al. 2003, 2004; Hurwitz et al. 2003; Susswein et al. 1996). <i>Aplysia</i> interneuron L10 is cholinergic and makes excitatory connections (Koester and Alevizos 1989), for example, recruiting neurons R25/L25 to burst and trigger respiratory pumping (Byrne 1983; Koester 1989) as well as LUQ cells projecting to the kidney (Koester and Alevizos 1989).

Our results suggest cholinergic ionotropic receptors are capable of producing extended bursting in bag cell neurons. Although we have found no evidence of metabotropic cholinergic receptors, second messenger production associated with maintenance of the afterdischarge may arise because of Ca²⁺ influx or the autocrine effect of certain bag cell neuron peptides (Kaczmarek et al. 1978; Redman and Berry 1993; Wayne et al. 1999). Ca²⁺ influx may occur directly through the cholinergic
ionotropic receptor or via voltage-gated Ca\textsuperscript{2+} channels recruited during the acetylcholine-induced depolarization. Elevated intracellular Ca\textsuperscript{2+} has the potential to activate some of the various nonselective cation channels that drive the afterdischarge (Gardam and Magoski 2009; Geiger et al. 2009; Lupinsky and Magoski 2006; Magoski 2004; Magoski and Kaczmarek 2005; Wilson et al. 1996). Finally, the afterdischarge can be sustained by the acetylcholine-evoked depolarization opening bag cell neuron persistent Ca\textsuperscript{2+} current (Tam et al. 2009).

Multiple mechanisms for initiation of the afterdischarge. Brown et al. (1989) showed that in addition to pleuroabdominal connective stimulation, the afterdischarge can be initiated by activating pleural ectopic bag cell neurons (as opposed to cerebral ectopic bag cell neurons). This appears to be chemically mediated, since electrical coupling is not evident between pleural ectopic neurons and abdominal cluster neurons. Interestingly, these authors provide the only report of an afterdischarge being generated by intracellular stimulation of a single bag cell neuron in the cluster. In addition, application of atrial gland extract to the cerebral ganglia can induce afterdischarges; however, this is likely polysynaptic, since it only manifests when applied to the cerebral ganglia with an intact pleuroabdominal connective, and atrial gland extract has no effect on cultured bag cell neurons (Strumwasser et al. 1980).

These alternative means may account for those rarer occasions where we failed to observe acetylcholine-induce refractoriness or pharmacological block of the afterdischarge. Furthermore, different external stimuli, such as mating or chemical signals from an egg mass, can cause afterdischarges (Begnoche et al. 1996). Given the importance to species propagation, a diversity of mechanisms for afterdischarge initiation and egg laying is not surprising. It is testament to the survival strategies involved in reproduction.

In summary, acetylcholine evokes an afterdischarge in the intact bag cell cluster. This is likely due to presynaptic release from neurons in the cerebral ganglion and opening of an ionotropic receptor.

ACKNOWLEDGMENTS

We thank S. L. Smith and H. M. Fuller for technical assistance.

GRANTS

S. H. White held a McLaughlin/Bracken Scholarship, and N. S. Magoski holds a Canadian Institutes of Health Research (CIHR) New Investigator Award. This work was supported by a CIHR operating grant to N. S. Magoski.

DISCLOSURES

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