Acetylcholine-evoked afterdischarge in *Aplysia* bag cell neurons

Sean H. White and Neil S. Magoski
Department of Biomedical and Molecular Sciences, Queen’s University, Kingston, Ontario, Canada

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White SH, Magoski NS. 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 a prolonged depolarization (Andrew and Dudek 1983; Burgoyne 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 either 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 (15.33 mg/ml; 165859; 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 at 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 poly styrene tissue culture dishes (430165; 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, Madison, WI, or 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/dispepsin (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 ml/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 antagonists, 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 (1–2-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); gamma butyrolactone (B1256; Sigma-Aldrich); oxotremorine sesquifumarate (O9126; Sigma-Aldrich); neostigmine bromide (N961; Sigma-Aldrich); nicotine (N0257; Sigma-Aldrich); oxytoxine sesquisulfurate (O9126; Sigma-Aldrich); Phe-Met-Arg-Phe amide (FMRFamide; P4898; Sigma-Aldrich); serotonin (H9523; Sigma-Aldrich); strychnine (S0532; SigmaAldrich); and tetramethylammonium chloride (TMA; T91526; 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 \(\sim 10\)-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 ± 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) 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 Gerschenfeld 1961). As such, at least four cholinergic receptor types are believed to exist: two distinctive \(\text{Cl}^-\) conductances, one rapidly desensitizing and the other slowly desensitizing, mediate the hyperpolarizing responses (Gardner and Kandel 1977; Kehoe 1972c; Kehoe and McIntosh 1998); the 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 \(\mu M\) 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 \(\mu M\) 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 the effect of glycine. As shown in Fig. 2, E and F, no detectible current was produced by glycine \((n = 8)\).

Interestingly, application of up to 3 mM nicotine \((n = 9)\) failed to replicate the acetylcholine-induced current and produced a significantly smaller current density of \(-0.12 \pm 0.01\) pA/pF, compared with \(-5.8 \pm 0.7\) pA/pF for 1 mM acetylcholine (Fig. 2F). TMA, which mimics the excitatory action of acetylcholine without inducing metabotropic affects in both other Aplysia neurons (Ascher et al. 1978a) and the homologous caudodorsal cells of Lymnaea (ter Maat and Lodder 1980), was the only compound to evoke a current of similar density to acetylcholine \((n = 20)\) (Fig. 2F). The general cholinergic agonist carbachol \((n = 6)\) (Schwartz et al. 1982), as well as the specific \(\alpha 7\) nicotinic receptor agonist choline \((n = 9)\) (Alkondon et al. 1997; Kehoe 1972c; Papke et al. 1996), also failed to induce a response (Fig. 2F).

Even though serotonin (5-HT) is able to block the afterdischarge, previous attempts at observing 5-HT-induced currents in cultured bag cell neurons had not been successful (Jennings et al. 1981). In our cells, 5-HT never induced an inward current but occasionally produced an outward current, and only through pressure application. As such, the average current density of \(0.21 \pm 0.13\) pA/pF \((n = 14)\) largely includes neurons where 5-HT provoked no current (Fig. 2F).

**Acetylcholine-induced current is not dependent on G proteins.** 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 GDP\(\beta S\) (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 GDP\(\beta S\) (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 GDP\(\beta S\) in the pipette (Fig. 3B). As a positive control, the outward current at \(-40\) mV produced by pressure-applied FMRFamide (500 \(\mu M\)), a known metabotropic agonist in molluscs (Brezina 1988; Fisher et al. 1993; Piomelli et al. 1987a, 1987b), was blocked by GDP\(\beta S\) (Fig. 3, C and D). The summary data of FMRFamide following GDP\(\beta S\) 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. 1995; 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.
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, nAChR 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 (Coggleshall 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.
(Kaczmarek et al. 1978). 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. 8, A and C, left), which became refractory to subsequent stimulation. Pretreatment with both 100 μM mecamylamine and 1 μM...
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). 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).

**Fig. 5. Differential voltage dependence of nicotinic receptor antagonist block of the ACh-induced current.** A: following the same protocol described in Fig. 4, but at a holding potential of −30 mV, the second Ach (1 mM) current is 68.0 ± 4.9% of the first in control conditions. B: a 10-min application of hexamethonium fails to alter the ACh-induced response at −30 mV (56.7 ± 5.5% remaining current at −30 mV vs. 16.6 ± 5.2% at −60 mV). Note that the percent current remaining at −30 mV in hexamethonium is similar to that of the control experiments at the same voltage. C: mecamylamine inhibited the ACh-induced response at −30 mV; however, by a reduced factor of 3 (22.5 ± 8.8% remaining current at −30 mV vs. 7.7 ± 3.3% at −60 mV). D: α-conotoxin ImI blocks the ACh-induced current equally well (14.0 ± 3.1 and 17.1 ± 8.5% current remaining at −60 and −30 mV, respectively). E: as at −60 mV, the coapplication of mecamylamine and α-conotoxin ImI eliminates the current at −30 mV. F: summary data comparing each antagonist at both resting and depolarized (−30 mV) potentials show the percent remaining current reaches the level of significance for hexamethonium (*P < 0.0005, 1-tailed Mann-Whitney U-test) and mecamylamine (*P < 0.05, 1-tailed Mann-Whitney U-test), but not for control or α-conotoxin ImI.
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 CIC− 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 1968; McCaman and Dewhurst 1970). 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 Cotrell 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-
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 Iml. 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 *Lymnaea* 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 Mpitssos 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 Ca$^{2+}$-dependent manner (Yamashita and Isa 2003). Acetylcholine evokes plateau potentials and bursting in *Lymnaea* 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 *Aplysia* feeding central pattern generator (Dembrow et al. 2003, 2004; Hurwitz et al. 2003; Susswein et al. 1996). *Aplysia* 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 Ca$^{2+}$ influx or the autocrine effect of certain bag cell neuron peptides (Kaczmarek et al. 1978; Redman and Berry 1993; Wayne et al. 1999). Ca$^{2+}$ influx may occur directly through the cholinergic...
ionotropic receptor or via voltage-gated $Ca^{2+}$ channels recruited during the acetylcholine-induced depolarization. Elevated intracellular $Ca^{2+}$ 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 $Ca^{2+}$ 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.

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**DISCLOSURES**

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

Author contributions: S.H.W. and N.S.M. conception and design of research; S.H.W. performed experiments; S.H.W. and N.S.M. analyzed data; S.H.W. and N.S.M. interpreted results of experiments; S.H.W. and N.S.M. prepared figures; S.H.W. drafted manuscript; S.H.W. and N.S.M. edited and revised manuscript; S.H.W. and N.S.M. approved final version of manuscript.

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