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Tyrosine Phosphorylation Determines Afterdischarge Initiation by Regulating an Ionotropic Cholinergic Receptor

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Abstract—Changes to neuronal activity often involve a rapid and precise transition from low to high excitability. In the marine snail, *Aplysia*, the bag cell neurons control reproduction by undergoing an afterdischarge, which begins with synaptic input releasing acetylcholine to open an ionotropic cholinergic receptor. Gating of this receptor causes depolarization and a shift from silence to continuous action potential firing, leading to the neuroendocrine secretion of egg-laying hormone and ovulation. At the onset of the afterdischarge, there is a rise in intracellular Ca^{2+}, followed by both protein kinase C (PKC) activation and tyrosine dephosphorylation. To determine whether these signals influence the acetylcholine ionotropic receptor, we examined the bag cell neuron cholinergic response both in culture and isolated clusters using whole-cell and/or sharp-electrode electrophysiology. The acetylcholine-induced current was not altered by increasing intracellular Ca^{2+} via voltage-gated Ca^{2+} channels, clamping intracellular Ca^{2+} with exogenous Ca^{2+} buffers, or activating PKC with phorbol esters. However, lowering phosphotyrosine levels by inhibiting tyrosine kinases both reduced the cholinergic current and prevented acetylcholine from triggering action potentials or afterdischarge-like bursts. In other systems, acetylcholine receptors are often modulated by multiple signals, but bag cell neurons appear to be more restrictive in this regard. Prior work finds that, as the afterdischarge proceeds, tyrosine dephosphorylation leads to biophysical alterations that promote persistent firing. Because this firing is subsequent to the cholinergic input, inhibiting the acetylcholine receptor may represent a means of properly orchestrating synaptically induced changes in excitability.

Key words: nicotinic receptor, calcium, protein kinase C, tyrosine phosphorylation, tyrosine, phosphatase.

INTRODUCTION

Switching a neuron from quiescent to bursting requires an initial, suprathreshold stimulus followed by recruitment of one or more sustained inward currents to preserve depolarization (Major and Tank, 2004; Bouhadfane et al., 2013; Tsuruyama et al., 2013). In some instances, second messengers and kinases are enlisted to regulate specific currents (Egorov et al., 2002; Canepari and Ogden, 2003; Mejia-Gervacio et al., 2004; Li et al., 2007; Yan et al., 2009). A well-characterized example is the bag cell neurons from the anaspedean marine snail, *Aplysia californica* (Kupfermann et al., 1966; Zhang and Kaczmarek, 2008). These neuroendocrine cells serve as command neurons for reproduction by secreting egg-laying hormone into the blood during a prolonged afterdischarge (Arch, 1972; Chiu et al., 1979; Rothman et al., 1983; Michel and Wayne, 2002). The afterdischarge is triggered by a brief (1–10 s) cholinergic input, followed by depolarization and a lengthy period of collective firing with a distinct fast- (~5 Hz for ~1 min) and slow- (~1 Hz for ~30 min) phase (Kupfermann and Kandel, 1970; Ferguson et al., 1989; White and Magoski, 2012). Upon cessation of the afterdischarge, the neurons enter an ~18-h refractory period, where synaptic input can still evoke action potentials, but prolonged bursting does not occur (Kaczmarek et al., 1978). The bag cell neurons are located in two, electrically coupled clusters, and can be examined either at the level of the network (in the nervous system or as isolated clusters) or the cell (as individual cultured neurons) (Kaczmarek et al., 1979; Kauer and Kaczmarek, 1985).

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Abbreviations: Ach, acetylcholine chloride; ATP, adenosine 5′-triphosphate 2Na·H_2O; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; Ca^{2+}-Cs^+-TEA-ASW, artificial seawater with tetraethylammonium substituted for Na^+ and Cs^+ for K^+; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol bis (aminoethylether) tetraacetic acid; GTP, guanosine 5′-triphosphate Na·H_2O; HEPES, N-2-hydroxyethlpiperazine-N′-2-ethanesulphonic acid; nASW, normal artificial seawater; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PP1, 1-(1,1-dimethylethyl)-1-(4-methylphenyl)-1H-pyrazolo [3,4-d]pyrimidin-4-amine; tcASW, tissue culture artificial seawater; TEA, tetraethylammonium; YEEI peptide, (NH_2-Glu-Pro-Gln-Tyr (PO_3H_2)-Glu-Glu-Ile-Pro-Ile-Tyr-Leu-OH.

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We previously established that acetylcholine initiates the afterdischarge by opening an ionotropic receptor (White and Magoski, 2012). The resultant depolarization is then maintained by activation of a persistent Ca^{2+} current and several non-selective cation currents (Hung and Magoski, 2007; Hickey et al., 2010; Sturgeon and Magoski, 2016). This includes a voltage-dependent cation channel opened by combination of depolarization, increased intracellular Ca^{2+} (which occurs as spiking begins), and phosphorylation by protein kinase C (PKC) (which is triggered 1–2 min into the afterdischarge) (Conn et al., 1989; Wilson et al., 1996, 1998; Wayne et al., 1999; Magoski et al., 2002; Lupinsky and Magoski, 2006). In addition, tyrosine dephosphorylation of the voltage-dependent cation channel promotes the physical association of PKC with the channel and a change in gating mode (from intermittent bursts to continuously active) (Wilson and Kaczmarek, 1993; Magoski and Kaczmarek, 2005).

A brief, focal application of acetylcholine depolarizes bag cell neurons via a concomitant inward current due to activation of cys-loop-type ionotropic cholinergic receptors (White and Magoski, 2012; White et al., 2014). The present study examines whether the cholinergic response is influenced by those second messengers and kinases/phosphatases that accompany the afterdischarge. Of the signaling pathways considered above, only tyrosine dephosphorylation impacts the cholinergic response; specifically, we observe a reduction in the acetylcholine-induced current, depolarization, and afterdischarge-like firing. Given that tyrosine dephosphorylation is thought to occur later in the afterdischarge (Wilson and Kaczmarek, 1993; Magoski and Kaczmarek, 2005), this would serve to dampen the effect of acetylcholine at a time-point where synaptic excitation is no longer needed.

EXPERIMENTAL PROCEDURES

Animals and cell culture

Aplysia californica (a hermaphroditic marine snail) were purchased as adults from Marinus Inc (Long Beach, CA, USA) at a weight of 150–500 g, and maintained at 15 °C on a 12/12-h light/dark cycle in an ~300-l aquarium filled with circulating, aerated artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH, USA). Animals were given Romaine lettuce 5 times a week. The Queen's University Animal Care Committee (Protocol number 100323-Magoski-2012) approved all procedures.

To make primary cultures of individual bag cell neurons, an injection of isotonic $MgCl_2$ (~50% of body weight) was given to anaesthetize the *Aplysia*. The abdominal ganglion was then dissected out and incubated in neutral protease (13.33 mg/ml; 165859; Roche Diagnostics, Indianapolis, IN, USA) dissolved in tissue culture artificial seawater (tcASW) (composition in mM: 460 NaCl, 10.4 KCl, 11 $CaCl_2$, 55 $MgCl_2$, 15 N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (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. Subsequently, the ganglion was rinsed for 1 h in fresh tcASW, and fine scissors and forceps were used to microdissect the bag cell neuron clusters from adjacent connective tissue. These de-sheathed clusters were then either placed in tcASW in a 14 °C incubator and used for intracellular recording the next day (see *Experimental procedures, Sharp-electrode current-clamp recording from bag cell neurons in culture and isolated clusters* for details) or bag cell neurons were gently triturated from them into tcASW-containing 35 × 10-mm polystyrene tissue culture dishes (430165; Corning, Corning, NY, USA or 353001; Falcon Becton-Dickinson, Franklin Lakes, NJ, USA) with a fire-polished Pasteur pipette. Cultures were kept at 14 °C and used for experiments 1–3 d later. Salts were supplied by Fisher Scientific (Ottawa, ON, Canada), ICN (Aurora, OH, USA), or Sigma–Aldrich (St. Louis, MO, USA).

Whole-cell voltage-clamp recording from cultured bag cell neurons

Tight-seal, whole-cell voltage-clamp recordings were made from cultured bag cell neurons at room temperature (20–22 °C) with an EPC-8 amplifier (HEKA Electronics; Mahone Bay, NS, Canada). Borosilicate glass capillaries (1.5-mm external-, 1.2-mm internal-diameter; TW150F-4; World Precision Instruments; Sarasota, FL, USA) were used to pull microelectrodes, which had a resistance of 1–2 MΩ following fire-polishing and filling with either intracellular solution (see below). Pipette junction potentials were set to 0 mV before seal formation; upon making a seal, the capacitance of the pipette was canceled and, once whole-cell mode was established, the cellular membrane capacitive was canceled. A compensation of 80% was achieved for the 3–5 MΩ series resistance. The EPC-8 Bessel filter was used to filter the current signal to 1 kHz before sampling at 2 kHz with the Clampex acquisition program of pCLAMP (v10.0; Molecular Devices; Sunnyvale, CA, USA).

Most voltage-clamp recordings employed normal artificial seawater (nASW; same composition as tcASW but lacking the glucose and antibiotics) in the bath, and a K^+-based intracellular solution in the recording pipette (composition in mM: 500 K-aspartate, 70 KCl, 1.25 $MgCl_2$, 10 HEPES, 11 glucose, 10 glutathione, 5 ethyleneglycol bis (aminoethylether) tetraacetic acid (EGTA), 5 adenosine 5′-triphosphate $2Na{\cdot}H_2O$ (ATP) (A3377; Sigma–Aldrich), and 0.1 guanosine 5′-triphosphate $Na{\cdot}H_2O$ (GTP) (G8877; Sigma–Aldrich); pH 7.3 with KOH). Based on a calculation using WebMaxC (http://web.stanford.edu/~cpatton/webmaxcS.htm), 3.75 mM $CaCl_2$ was added to this solution so to set the free Ca^{2+} concentration at 300 nM, which corresponds to the resting intracellular Ca^{2+} as determined in prior studies (Loechner et al., 1992; Fisher et al., 1994; Magoski et al., 2000). In one case, EGTA was left out of the intracellular solution and no Ca^{2+} was added. Also, for low intracellular Ca^{2+}, 10-mM 1,2-bis(*o*-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) (A4926; Sigma–Aldrich) was substituted for EGTA, resulting in a free Ca^{2+} concentration of 30 nM. The junction potential

of the intracellular solution *vs* nASW was determined to be 15 mV and subsequently subtracted off-line.

In one set of experiments, voltage-gated Ca^{2+} currents were recorded using Ca^{2+}-Cs^{+}-TEA-ASW, where Na^{+} was replaced with tetraethylammonium (TEA) and K^{+} with Cs^{+} (composition in mM: 460 TEA-Cl, 10.4 CsCl, 55 $MgCl_2$, 11 $CaCl_2$, and 15 HEPES; pH 7.8 with CsOH). A Cs^{+}-based internal solution with no added Ca^{2+} was used (composition in mM: 70 CsCl, 10 HEPES, 11 glucose, 10 glutathione, 5 EGTA, 500 aspartic acid, 5 ATP, and 0.1 GTP, pH 7.3 with CsOH). Leak subtraction was carried out on-line with a *P*/4 protocol during Ca^{2+} current recording. From −60 mV, subpulses that were opposite in polarity and one-quarter the size of the 200-ms test pulses were delivered at 500-ms intervals, with a settling time of 100 ms prior to the actual test pulses (see *Results* for details). Off-line subtraction was again used to eliminate a junction potential of 20 mV.

Sharp-electrode current-clamp recording from bag cell neurons in culture or isolated clusters

Sharp-electrode, bridge-balanced current-clamp recordings were carried out at room temperature (20–22 °C) from cultured bag cell neurons in nASW or de-sheathed bag cell neuron clusters in tcASW using an AxoClamp 2B amplifier (Molecular Devices). Borosilicate glass capillaries (1.2-mm external-, 0.9-mm internal-diameter; TW120F-4; World Precision Instruments) were used to pull microelectrodes, which were filled with 2-M K^{+}-acetate plus 10-mM HEPES and 100-mM KCl (pH 7.3 with KOH) for a resistance of 5–20 MΩ. The Axoclamp Bessel filter was used to filter the voltage signal to 3 kHz before sampling at 2 kHz as per whole-cell voltage-clamp with Clampex. When necessary, current was injected into the neuron using Clampex.

Photomicroscopy

Bright-field photomicrographs of cultured bag cell neurons or isolated bag cell neuron clusters were taken with a Nikon TS100-F inverted microscope (Nikon; Mississauga, ON, Canada) furnished with Nikon Plan Fluor 4× (numerical objective (NA) = 0.13) or 10 × (NA = 0.3) objectives. The tissue was excited with a 30-W halogen lamp and images (1392 × 1040 pixels) acquired with a Pixelfly USB camera (PCO-TECH, Photon Technology International; London, ON, Canada) controlled by the Micro-Manager (v1.4.5; https://micro-manager.org) plugin for ImageJ (v1.44n9; https://imagej.nih.gov/ij/) with 100–200 ms exposure times.

Drug application and reagents

Acetylcholine was delivered onto the soma, via an unpolished whole-cell pipette (1–2-μm bore) positioned 50 μm away, by pressure-ejection for 2 s at 75–150 kPa with a PMI-100 pressure ejector (Dagan; Minneapolis, MN, USA). As carried out by Fisher et al. (1993) and ourselves (White and Magoski 2012; White et al., 2014, 2016), the neurons were not typically superfused during pressure-application. To curtail leakage and any receptor desensitization, the pipette was removed from the bath right after each ejection. Usually, concentrated stock solutions of other drugs were delivered by pipetting a small volume directly into the bath at least 10 min prior to acetylcholine pressure-application, with incubation in DMSO (for hydrophobic substances) or water (for hydrophilic substances) serving as control. That stated, a few experiments required genistein or genistin to be superfused over cultured neurons at final concentration. A square-barreled glass pipette (~500-μm bore) was positioned with a micromanipulator 300–500 μm from the soma. This pipette continuously flowed nASW over the neuron, which was switched to drug-containing saline by activating a stopcock to select between various gravity-driven reservoirs. Acetylcholine was then pressure-ejected directly into the superfusion stream (see *Results* for details).

Stocks of the drugs were dissolved in water or DMSO and frozen at −20 °C, then diluted to a final concentration in either extracellular saline or intracellular solution: acetylcholine chloride (A6625; Sigma–Aldrich), C2-ceramide (BML-SL100-0005; Biomol/Enzo Life Sciences; Plymouth Meeting, PA, USA) and YEEI peptide (NH_2-Glu-Pro-Gln-Tyr(PO_3H_2)-Glu-Glu-Ile-Pro-Ile-Tyr-Leu-OH; BML-P300-0001; Biomol/Enzo Life Sciences) all required water as a vehicle, whereas phorbol 12-myristate 13-acetate (PMA) (P8139; Sigma–Aldrich), genistein (G6649; Sigma–Aldrich), genistin (G8097; Sigma–Aldrich), and 1-(1,1-dimethyle thyl)-1-(4-methylphenyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine (PP1) (1398; Tocris Bioscience; Minneapolis, MN, USA) all required DMSO. The final concentration of DMSO was ≤0.2%, which here or in prior studies was found to have no effect on bag cell neuron membrane potential, holding current, Ca^{2+} current, or intracellular Ca^{2+} (Magoski et al., 2000; Kachoei et al., 2006; Geiger and Magoski, 2008; Tam et al., 2009, 2011; Hickey et al., 2010; Groten et al., 2013, 2016; Groten and Magoski, 2015; Sturgeon and Magoski, 2016).

Analysis

The amplitude and time course of acetylcholine-induced changes in membrane potential or current, as well as Ca^{2+} current evoked by test voltages, were measured with the Clampfit analysis program of pCLAMP. Just before any change to current or voltage, two cursors were positioned, while at the peak response, two more cursors were also placed. The average current or voltage was then determined between the paired cursors, and the difference between the mean baseline and mean peak values was considered as the maximal response. In some cases, the cellular capacitance from the EPC-8 whole-cell capacitance compensation circuitry was divided into the current so to normalize the response to cell size. When the neuron fired action potentials during a depolarizing response under current-clamp, Clampfit was used to generate all-points histograms for before and after acetylcholine application. The largest peak of the resulting histograms was fit in Clampfit with a Gaussian function by the least-squares method plus a simplex search, and taken as

the average membrane potential during these time periods. For display only, the Clampfit Gaussian filter was used to filter some acetylcholine-induced current traces off-line at 20–80 Hz. Because the kinetics of these currents was slow, the secondary filtering did not alter the amplitude. That stated, no subsequent filtering was employed for display of either voltage-gated Ca^{2+} currents or acetylcholine-elicited changes to membrane potential.

Summary data are presented as mean ± standard error of the mean (for normally distributed data) or median with all of the contributing data points (for non-parametrically distributed data). Prism (v3.1; GraphPad Software Inc; La Jolla, CA, USA) was used for statistical analysis. Normality was tested with the Kolmogorov–Smirnov method. Student's paired or unpaired *t*-test was used to test for differences between two groups of normally distributed data. The Mann–Whitney *U*-test (for unpaired data) or the Wilcoxon matched-pairs signed rank test (for paired data) was used to test differences between two groups of non-parametrically distributed data. To test whether the mean differed between three groups, an ordinary analysis of variance (ANOVA) with a Bonferroni multiple comparisons test was used. The *t*-, *F*-, *U*-, or *Z*-statistic are given, along with the degrees of freedom (in lower case). Means were considered significantly different if the two-tailed *p*-value was ≤ 0.05.

RESULTS

Depolarization-induced Ca^{2+} influx does not alter the acetylcholine-induced current

Elevated intracellular Ca^{2+} is reported to lessen acetylcholine-induced currents in rat chromaffin cells as well as habenular and hippocampal neurons (Khiroug et al., 1997, 1998, 2003; Guo and Lester, 2007). Considering that Ca^{2+} influx greatly elevates bag cell neuron cytosolic Ca^{2+} at the start of the afterdischarge (Fisher et al., 1994), while acetylcholine is likely still present in the synaptic cleft, we initially explored the influence of Ca^{2+} on the cholinergic response. Cultured bag cell neurons were bathed in normal artificial seawater (nASW) and whole-cell voltage-clamped using a K^+-based intracellular solution (see *Experimental procedures*, *Whole-cell voltage-clamp recording from cultured bag cell neurons* for details). Voltage-gated Ca^{2+} channels were activated with a stimulus mimicking the fast-phase of the afterdischarge, *ie*, from a holding potential of −60 mV, neurons were given a 5-Hz, 1-min train-stimulus of 75-ms square pulses to 0 mV. In order to not impact the diffusion of Ca^{2+} once it had entered the cell, the intracellular solution lacked the standard 5 mM of the slow Ca^{2+} buffer, EGTA (Naraghi, 1997). Our previous work, using neurons whole-cell loaded with the Ca^{2+}-sensitive dye, fura, demonstrated that this train-stimulus evoked a 200–300% increase in cytosolic Ca^{2+} which recovered with a time constant of ~40 s (Groten et al., 2013).

Acetylcholine was pressure-ejected for 2 s onto the soma at a concentration of 1 mM (Fig. 1A, *right*) (see *Experimental procedures*, *Drug application and reagents* for details). As per our prior efforts (White and Magoski, 2012; White et al., 2014), any neuron-to-neuron variability in the acetylcholine response was accounted for by comparing the magnitude of two acetylcholine-induced currents separated by at least 10 min. In control conditions, without exposure to the train-stimulus ($n = 5$), the peak magnitude of the second cholinergic current was ~55% of the first (Fig. 1A, *left*, *C*). This decrease is the result of desensitization of the ionotropic receptor (White and Magoski, 2012). For the experimental group, the train-stimulus was given between the two acetylcholine applications; specifically, the second acetylcholine-induced current was elicited 5–10 s after the train-stimulus, thus ensuring the cholinergic current occurred while intracellular Ca^{2+} was still well elevated. For neurons exposed to the train-stimulus ($n = 6$), the Ca^{2+} rise failed to alter the acetylcholine response, resulting in an ~60% current remaining (Fig. 1B, C). On average, this was not significantly different from control neurons which did not experience Ca^{2+} influx between the acetylcholine applications (Fig. 1C).

If Ca^{2+} influx were to impact the cholinergic current, this would only occur once the afterdischarge had started. Thus, we also tested whether altering the basal intracellular Ca^{2+} concentration could influence afterdischarge initiation by affecting the acetylcholine-induced current prior to Ca^{2+} influx. Control neurons were intracellularly perfused (aka dialyzed) for 20 min via the whole-cell electrode with standard intracellular solution, which contained 5-mM EGTA and a free Ca^{2+} concentration set to 300 nM (see *Experimental procedures*, *Whole-cell voltage-clamp recording from cultured bag cell neurons* for details) ($n = 8$). This Ca^{2+} concentration is based on the resting intracellular Ca^{2+} measurements performed in previous work using either calibrated Ca^{2+}-sensitive microelectrodes or dyes (Loechner et al., 1992; Fisher et al., 1994; Magoski et al., 2000). A second group of neurons was also intracellularly perfused, but with an intracellular solution where EGTA was replaced with 10 mM of the relatively fast Ca^{2+} buffer, BAPTA (Naraghi, 1997), and the free Ca^{2+} set to 30 nM ($n = 7$). Neurons in both sets subsequently had 1-mM acetylcholine pressured applied for 2s onto the soma (Fig. 1D); the resulting cholinergic currents were then normalized to whole-cell capacitance and expressed as current density. Despite having a lower free Ca^{2+} concentration, the average response from the BAPTA group was not significantly different from the EGTA group (Fig. 1E).

PKC activation does not regulate the cholinergic current

Acetylcholine-induced currents in sympathetic ganglion and *Aplysia* R15 or RB-group neurons, as well as heterologously expressed $\alpha4\beta2$ nicotinic receptors, are modulated by PKC (Downing and Role, 1987; Papp and Hoyer, 1996; Fenster et al., 1999a). In bag cell neurons, PKC activity is elevated within two min of afterdischarge onset (Conn et al., 1989; Wayne et al., 1999). This regulates a voltage-dependent cation channel and leads to insertion of voltage-gated Ca^{2+} channels into the membrane (Strong et al., 1987; Wilson et al., 1998; Magoski

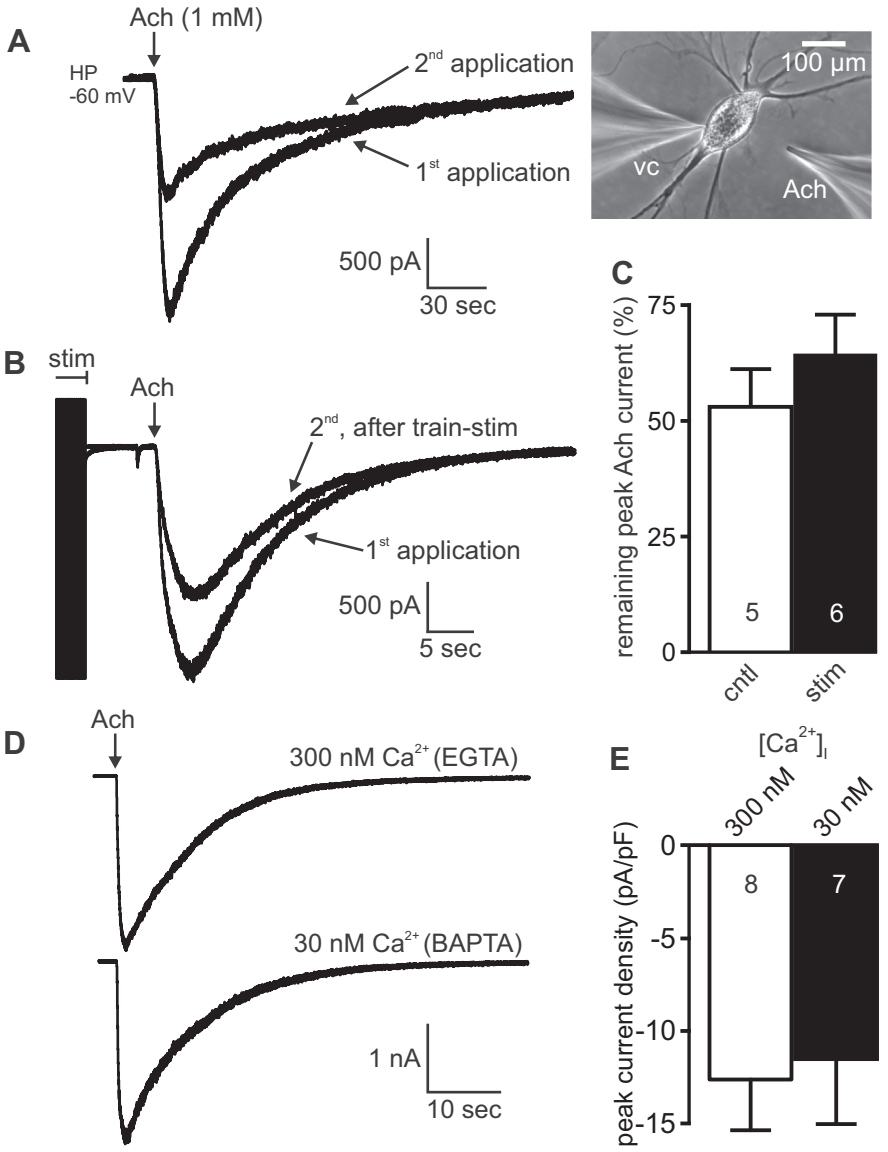


Fig. 1. The acetylcholine-induced current is not regulated by intracellular Ca^{2+}. (A) *Left panel*, a cultured bag cell neuron in nASW external saline is whole-cell voltage-clamped at −60 mV using standard K^+-based internal solution lacking the Ca^{2+} buffer, EGTA. An initial 2-s pressure-application of 1-mM acetylcholine (Ach, at *arrow*) almost immediately evokes a relatively large (~2-nA) inward current that peaks within 5 s of transmitter delivery. Ten min after the current returns to baseline, a second acetylcholine application generates a response of about half the amplitude of the first. The difference in amplitude is due to receptor desensitization (White and Magoski, 2012). Current traces overlaid for clarity. *Right panel*, photomicrograph of an *in vitro* bag cell neuron soma with sprouted neurites; the whole-cell voltage-clamp (vc) electrode is middle-left, while the acetylcholine (Ach) ejection pipette is lower-right. (B) In a different neuron, when intracellular Ca^{2+} is increased with a 5-Hz, 1-min train-stimulus of 75-ms voltage steps to 0 mV (stim, at bar) during the interval between acetylcholine applications, the second response is little under two-thirds of the first. For display, only the last 5 s of the train-stimulus is shown; the membrane current response during this time is the vertical bar of condensed deflections apparent on the left of the *2nd, after train-stim* trace. Pre and post train-stim traces overlaid for clarity. (C) Summary data indicate that the second peak acetylcholine-induced current is not significantly different when the train-stimulus is delivered between transmitter applications *vs* no intervening stimulus ($t_9 = 0.9377$, $p > 0.05$, unpaired Student's *t*-test). (D) Buffering intracellular Ca^{2+} to 30 nM with 10-mM BAPTA in the intracellular solution (*lower*), does not alter the acetylcholine-induced current when compared to a parallel control neuron where Ca^{2+} is buffered to 300 nM with 5-mM EGTA. The scale bars apply to both traces. (E) When peak acetylcholine-induced current is normalized to cellular capacitance, there is no significant difference in average current density between the intracellular Ca^{2+} concentrations ($t_{13} = 0.2508$, $p > 0.05$, unpaired Student's *t*-test).

and Kaczmarek, 2005; Zhang et al., 2008; Gardam and Magoski 2009). Hence, we chose to examine the effects of PKC on the bag cell neuron cholinergic current using the phorbol ester, PMA (Castagna et al., 1982), which has been shown to directly activate PKC in *Aplysia* sensory and bag cell neurons (DeRiemer et al., 1985a; Sossin and Schwartz, 1994).

Cultured bag cell neurons were whole-cell voltage-clamped at −60 mV and 1-mM acetylcholine pressure-ejected onto the soma for 2 s to evoke an initial current. For this and all subsequent whole-cell experiments, the intracellular solution contained 5-mM EGTA to buffer the Ca^{2+} to 300 nM. In control conditions, neurons were then exposed to DMSO ($n = 8$) for 20 min, followed by a second delivery of acetylcholine. This subsequent application resulted in a latter current that was ~60% of the initial current (Fig. 2A, C). For the experimental group of neurons, a 20-min exposure to 1-µM PMA ($n = 7$) between the first and second application of acetylcholine resulted in a latter current that was also ~60% of the initial current (Fig. 2B, C). There was no statistical difference between the average data for these conditions (Fig. 2C).

The cholinergic current is reduced by tyrosine kinase inhibitors

Tyrosine phosphorylation enhances and reduces, respectively, mammalian α7 and *Torpedo californica* nicotinic receptor-mediated currents (Hopfield et al., 1988; Charpantier et al., 2005). During the afterdischarge, one of the cation channels that drives the depolarization is inhibited by tyrosine phosphorylation (Magoski and Kaczmarek, 2005). Therefore, we sought to test the impact of altering tyrosine kinase activity on both the acetylcholine-induced current and depolarization in bag cell neurons.

Using cultured neurons whole-cell voltage-clamped at −60 mV, a 2-s pressure-application of 1-mM acetylcholine was delivered twice, with an interval of ~10 min. Between the first and second acetylcholine-evoked currents, either 20 µM of the src-family tyrosine kinase inhibitor, PP1, or the vehicle, DMSO, was delivered to the bath. PP1 is selective over other tyrosine kinases, such as the epidermal

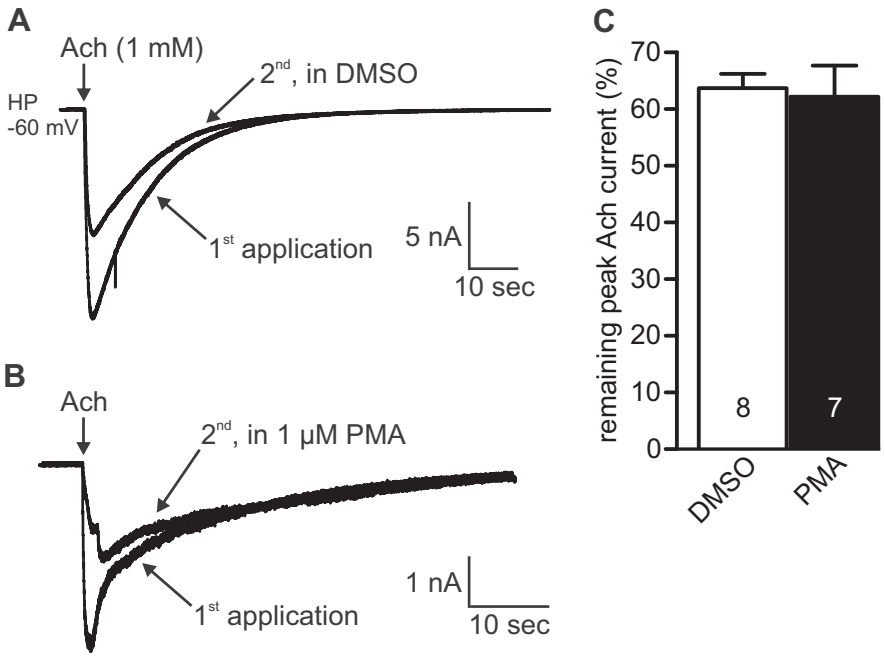


Fig. 2. A PKC activator does not alter the acetylcholine-induced current. (A) In a control cultured bag cell neuron voltage clamped at −60 mV, the first 2-s pressure-ejection of 1-mM acetylcholine (Ach, at *arrow*) elicits a large, rapid inward current. Following 20 min of DMSO, a second acetylcholine application yields a current that is nearly two-thirds of the initial response. Traces overlaid for clarity. (B) When 1 μM of the phorbol ester, PMA, is introduced into the bath for the 20 min between the two acetylcholine applications, the amplitude of the second cholinergic current is near half of the first. (C) Summary data show that cholinergic response is not significantly altered by the activation of PKC with PMA compared to the vehicle, DMSO (t_{13} = 0.2623, p > 0.05, unpaired Student's *t*-test). In both cases, the mean remaining peak acetylcholine-induced current is ∼60%.

growth factor receptor (EGFR) or Janus kinase, as well as protein kinase A (PKA) (Hanke et al., 1996). PP1 has also been shown to lower phosphotyrosine immunoreactivity and promote cation channel-PKC association in bag cell neurons, as well as inhibit tyrosine phosphorylation in *Aplysia* nervous tissue (Suter and Forscher, 2001 Magoski and Kaczmarek, 2005). With DMSO (n = 8), the peak of the latter current was ∼60% of the initial response (Fig. 3A, C). This was significantly different from PP1 (n = 9), which led to the second current being ∼25% of the first current (Fig. 3B, C).

Because reducing phosphotyrosine levels with PP1 decreased the cholinergic current, we also tested the effect of the general tyrosine kinase antagonist, genistein, and its inactive form, genistin, as a control. Genistein blocks both EGFR and src, but fails to act on PKA, PKC, or phosphorylase kinase (Akiyama et al., 1987). Genistein was previously shown to inhibit the related pentameric cys-loop γ-aminobutyric acid receptor in both cerebellar granule cells and forebrain neurons (Jassar et al., 1997; Balduzzi et al., 2002). For *Aplysia* sensory neurons, genistein prevents certain serotonin-induced changes, including enhanced excitability, increased glutamate uptake, and PKC translocation, (Purcell et al., 2003; Khabour et al., 2004; Nagakura et al., 2010). Genistein also impedes serotonin-mediated reversal of depressed sensory to motor neuron synapses as well as a type of intermediate-term synaptic facilitation brought about by serotonin and activity (Shobe et al., 2009; Nagakura et al., 2010) In bag cell neurons, genistein is similar to PP1, in that it reduces phosphotyrosine staining, particularly as documented in growth cones (Suter and Forscher, 2001).

Due to concentrated stocks of genistin coming out of solution when applied as a bolus to the bath, for this particular set of experiments both genistein and genistin were instead superfused close to the recorded neuronal somata (see *Experimental procedures*, *Drug application and reagents* for details). Brief (100-ms) pressure-ejections of 1-mM acetylcholine were given twice, directly into the superfusion stream, with genistin or genistein present for ∼10 min in the period separating the two cholinergic currents. Under the control condition of 50-μM genistin (n = 5) (the maximal concentration attainable in nASW), the peak of the latter acetylcholine current was ∼70% of the initial response (Fig. 4A, D). On the other hand, when exposed to 100-μM genistein (n = 6), the second current was less than ∼10% of the first; the summary data showed this difference to be significant (Fig. 4B, D).

The acetylcholine-induced depolarization is diminished after tyrosine kinase inhibition

A drop in the cholinergic current following tyrosine kinase inhibition should manifest as a lessening of the ability of acetylcholine to evoke depolarization and action potential firing. To determine whether this was the case, the membrane potential of cultured bag cell neurons was monitored (see *Experimental procedures*, *Sharp-electrode current-clamp recording from bag cell neurons in culture and isolated clusters* for details) and the effects of genistein on the acetylcholine-induced depolarization assessed. Cultured bag cell neurons were sharp-electrode current-clamped and the membrane potential initially set at −60 mV using bias current. A concentration of 1-mM acetylcholine was pressure-ejected for 2 s twice, with 100-μM genistein being delivered to the bath between applications (n = 6). The first ejection served as control and elicited an ∼30 mV depolarization that was always accompanied by a small number of action potentials, ranging between 2 and 15 spikes (Fig. 4C, *left*, E). Following genistein, again from a set membrane potential of −60 mV, the second ejection of acetylcholine evoked an ∼20 mV depolarization that never resulted in spiking (Fig. 4C, *right*, E). When comparing control *vs* genistein, both the average depolarization magnitude and number of action

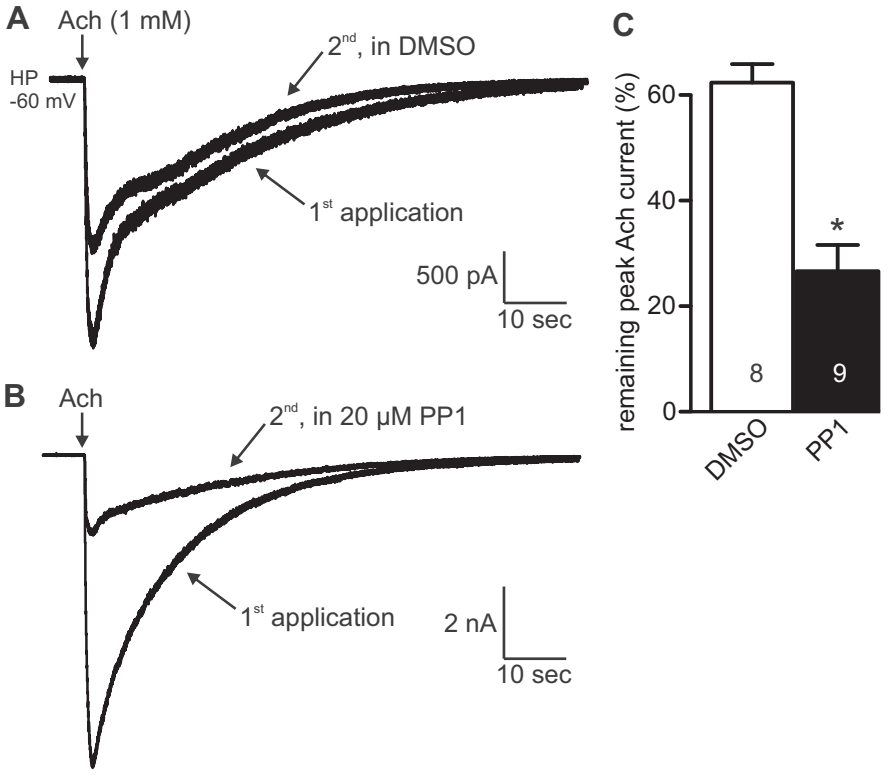


Fig. 3. The acetylcholine-induced current is attenuated by the src tyrosine kinase inhibitor, PP1. (A) Sequential 2-s pressure-ejections of 1-mM acetylcholine (Ach, at *arrow*), separated by an ~10-min interval, are delivered to a cultured bag cell neuron voltage clamped at −60 mV. Adding DMSO during the interval results in the current evoked by the second application being around two-thirds of the current generated by the first application. Traces overlaid for clarity. (B) A similar experiment as in *panel A*, with the exception of adding 20-μM PP1, a src tyrosine kinase inhibitor, to the bath for ~10 min after the first cholinergic current is elicited. The current brought about by the second application of acetylcholine is roughly one-quarter of that caused by the initial application. (C) Group data indicate that, compared to DMSO, treatment with PP1 significantly reduces the second peak acetylcholine-induced current to ~30% of the first ($^{*}t_{15} = 3.978$, $p \leq 0.002$, unpaired Student's *t*-test).

potentials produced by acetylcholine were significantly different (Fig. 4E, F).

The inability of acetylcholine to bring about action potential firing in bag cells neurons treated with tyrosine kinase inhibitors likely reflects a decline of the cholinergic current. However, it is possible that lowering phosphotyrosine levels also decreases excitability. To rule this out, we examined action potential threshold, input resistance, holding current, and voltage-gated Ca^{2+} current in cultured bag cell neurons. For threshold analysis, neurons were sharp-electrode current-clamped to a membrane potential of −60 mV with bias current. Neurons were given consecutive 50-ms current injections in 0.2-nA increments from 0.2 to 2nA; the current at which the first action potential occurred was taken as threshold (Fig. 5A, *left*). Subsequently, 100-μM genistein was introduced to the bath and 10 min later the current steps were injected a second time, again with the cell initially sitting at −60 mV. For both the control period and after genistein, the threshold was ~1 nA and not statistically different (n = 6) (Fig. 5A, *right*). Input resistance was measured over the same time-frame in these neurons by determining the steady-state voltage drop from −60 mV to a 1-s, 50-pA hyperpolarizing current injection. The input resistance was ~150 MΩ both before and after genistein, which failed to show a statistical difference ($n = 6$) (Fig. 5B).

To see whether tyrosine kinase inhibition altered steady-state holding current, neurons were whole-cell voltage-clamped at −60 mV and DMSO, 25-μM PP1, or 100-μM genistein bath applied. While neither DMSO ($n = 5$) nor PP1 ($n = 6$) had an appreciable impact on the current, genistein ($n = 18$) did bring about an inward current of ~−70 pA (Fig. 5C). Group data revealed that this genistein-induced change in holding current was significantly different from DMSO or PP1 (Fig. 5D). However, any inward current would be expected to facilitate rather than inhibit the acetylcholine-induced depolarization; thus, we did not pursue the nature of this conductance.

We also examined voltage-gated Ca^{2+} current, which not only contributes strongly to the upstroke of the bag cell neuron action potential, but can also influence excitability (DeRiemer et al., 1985b; Tam et al., 2009). Because genistein did not alter action potential threshold, which could be expected if tyrosine kinases regulated the Ca^{2+} current, we only examined the effects of PP1. Neurons were whole-cell voltage-clamped with a Cs^{+}-based intracellular solution and an extracellular solution where the Na^{+} and K^{+} were replaced with TEA and Cs^{+}, respectively (see *Experimental procedures*, *Whole-cell voltage-clamp recording from cultured bag cell neurons* for details). Neurons were treated for 10 to 60 min with either DMSO or 25-μM PP1, voltage-clamped at −60 mV, and stepped through to +60 mV with 200-ms pulses in 10-mV increments. For DMSO ($n = 5$), the onset of inward Ca^{2+} current occurred between −30 and −20 mV, followed by a peak between + 10 and +20 mV (Fig. 5E, *upper*). Ca^{2+} currents elicited in PP1 ($n = 7$) were very similar to those observed in DMSO (Fig. 5E, *lower*). When both data sets were normalized to cellular capacitance, to control for neuron size, and plotted against test potential, the resulting current–voltage relationships were largely the same with no statistical differences (Fig. 5F).

Attempts at enhancing tyrosine phosphorylation levels do not alter the cholinergic current

Knowing that the acetylcholine-induced current appears to be positively correlated to tyrosine phosphorylation, we sought to increase the magnitude of the cholinergic

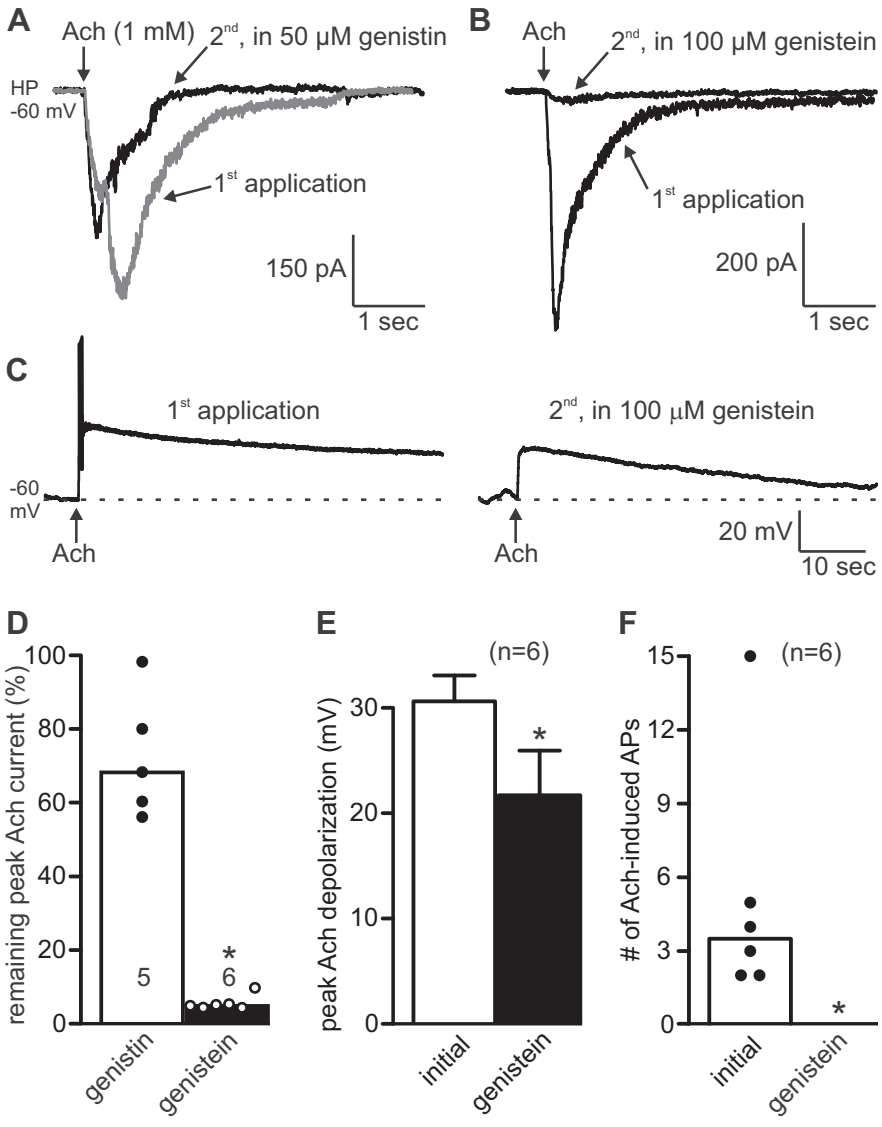


Fig. 4. The general tyrosine kinase inhibitor, genistein, attenuates both the acetylcholine-induced current and depolarization. (A) Acetylcholine is pressure-ejected (Ach, at *arrow*; 1 mM) for 100 ms twice into a stream of nASW superfused over the soma of a cultured bag cell neuron whole-cell voltage-clamped at −60 mV. The first application of acetylcholine (gray) evokes a modest but rapid current. After superfusing 50-μM genistin for 10 min, delivering acetylcholine again (black) results in a second current that is nearly three-quarters of the initial response. (B) Using the same protocol as in *panel A*, superfusing 100-μM of the general tyrosine kinase inhibitor, genistein, between acetylcholine ejections largely eliminates the second cholinergic current. Traces overlaid for clarity. (C) A cultured bag cell neuron is sharp-electrode current-clamped and the membrane potential set to −60 mV at the start of the experiment. An initial 2-s pressure-ejection of 1-mM acetylcholine depolarizes the neuron by > 30 mV, which is sufficient to generate action potentials (*left*); however, after adding 100-μM genistein to the bath for ∼10 min, another delivery of acetylcholine to the same neuron, again at −60 mV, leads to a smaller depolarization and no spiking (*right*). (D–F) Summary data show that the extent to which the peak current drops between the first and second acetylcholine application is significantly more with genistein compared to genistin ($^{*}U_{(5,6)} = 0.00$, $p \leq 0.003$, Mann–Whitney *U*-test). Similarly, subsequent to the introduction of genistein, there is a significant decrease in the acetylcholine-induced depolarization ($^{*}t_{10} = 1.820$, $^{*}p \leq 0.05$, paired Student's *t*-test) and number of evoked action potentials ($^{*}Z = 21.000$, $p \leq 0.04$, Wilcoxon matched-pairs signed rank test).

response by activating tyrosine kinases. Initially, the whole-cell pipette was used to intracellularly perfuse cultured bag cell neurons with either standard intracellular solution alone or one containing 100-μM of the src tyrosine kinase activator, YEEI peptide (Waksman et al., 1993), under voltage-clamp at −60 mV. In both conditions, ∼2 min after breakthrough, the cholinergic current was evoked by pressure-ejecting 1-mM acetylcholine onto the soma for 2 s. After 30 min of whole-cell recording, acetylcholine was applied again. In control conditions ($n = 6$), with just standard intracellular solution, the second acetylcholine-induced current was ∼60% of the first (Fig. 6A, C). This was similar to different neurons recorded using intracellular solution that included YEEI peptide ($n = 6$) (Fig. 6B, C). There was no significant difference between the average remaining cholinergic current of the control and YEEI peptide groups (Fig. 6C).

We also employed the membrane-permeable non-receptor tyrosine kinase activator, C2-ceramide (Ibitayo et al., 1998), which impairs growth cone formation in neurons from the pulmonate snail, *Helisoma* (Geddis and Rehder, 2003). Again, 1-mM acetylcholine was first pressure-ejected for 2 s, after which either DMSO (the vehicle) or 10-μM C2-ceramide was bath applied. Acetylcholine was then given a second time ∼20 min later. For DMSO ($n = 7$), the peak of the latter current was ∼50% of the initial response, while for C2-ceramide ($n = 10$) the remaining current was ∼45% of the first (Fig. 6D, E). Nevertheless, there was no statistical difference between these groups (Fig. 6F).

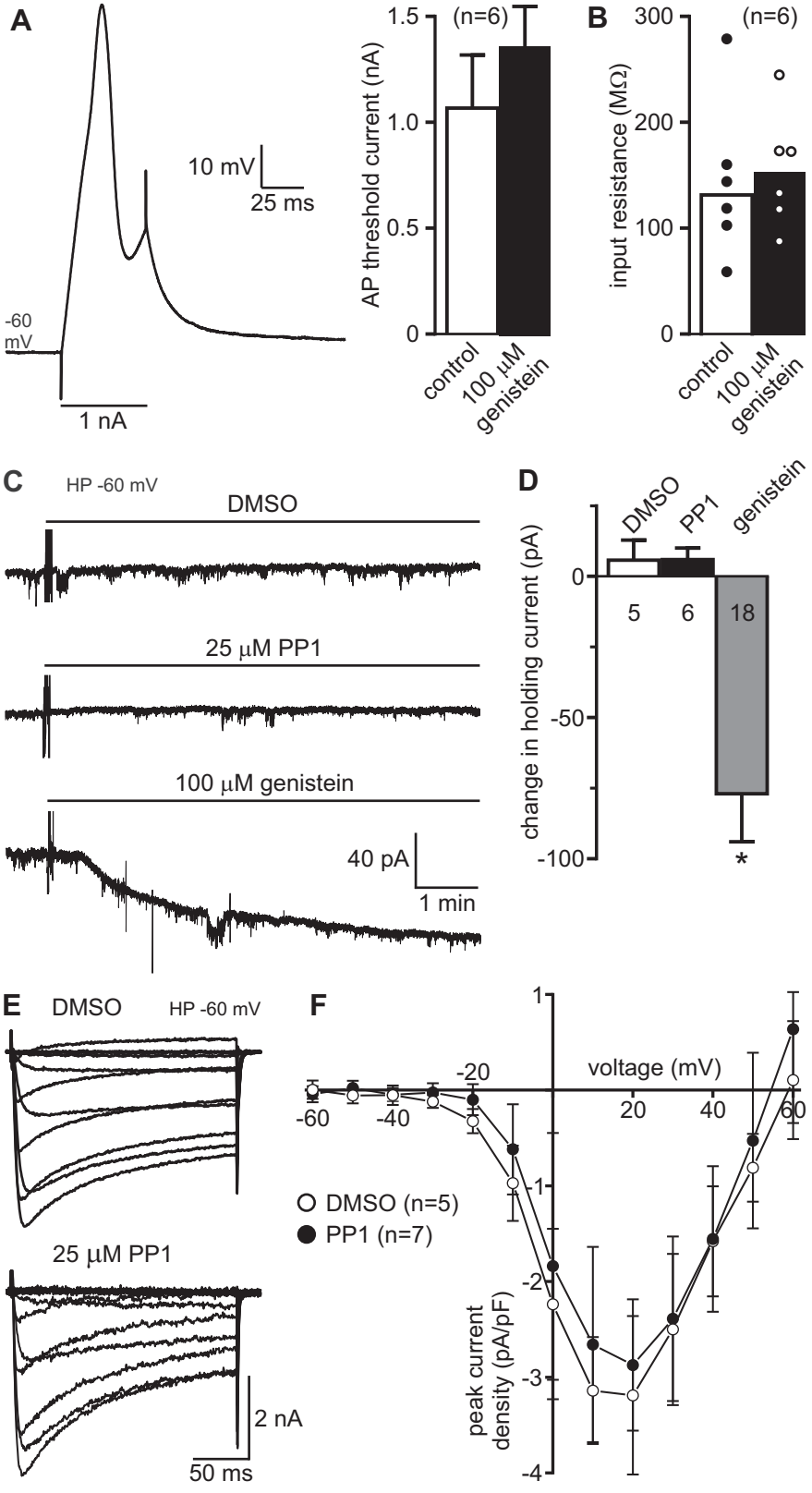
Acetylcholine-induced afterdischarge-like responses in isolated bag cell neuron clusters are suppressed by tyrosine kinase inhibition

Finally, we sought to ascertain if phosphotyrosine levels determine whether acetylcholine can elicit an afterdischarge in the bag cell neuron cluster. Although we previously found that continuous bath-application of acetylcholine can initiate an afterdischarge from the cluster while still in the intact abdominal ganglion, pressure-applying acetylcholine fails to evoke action potentials in that situation due to high amounts of both connective tissue and acetylcholine esterase (White and Magoski, 2012). Thus, for the present study, we used a modified version of an isolated cluster preparation first introduced by Kauer and Kaczmarek (1985). Specifically, abdominal ganglia

were dissected out, enzyme-treated overnight, and bag cell neuron clusters removed and separated from their surrounding connective tissue sheath. However, instead of being used to dissociate neurons into primary culture, the cluster was left overnight again to rest/recover, then used the next day for intracellular recording (see *Experimental procedures*, *Animals and cell culture* as well as *Sharp-electrode current-clamp recording from bag cell neurons in culture and isolated clusters* for details).

To demonstrate afterdischarge-like responses in these isolated, de-sheathed clusters, acetylcholine was pressure-applied while recording from a single bag cell neuron within the network (Fig. 7A). Neurons were monitored using sharp-electrode current-clamp and the membrane potential was initially set to −60 mV with bias current. In seven, separate bag cell neuron clusters, 1-mM acetylcholine was always pressure-ejected for 2 s onto one side of the cluster, while recording from a bag cell neuron on the opposite side (a distance of 200–300 μm away). All neurons depolarized and reached threshold to fire multiple action potentials. In four cases, an afterdischarge-like response was triggered, with a distinct fast- and slow-phase (Fig. 7B). In the other three instances, the neuron presented a more brief burst of spikes before slowly repolarizing back toward resting potential (Fig. 7C). The mean duration of the acetylcholine-induced burst firing for all neurons/clusters was 211.1 ± 91.05 s (n = 7). Given that bag cell neurons are electrically coupled in the cluster, the transfer of the cholinergic-induced depolarization and propagation throughout the network likely occurred through gap junctions (Kupfermann and Kandel, 1970; Blankenship and Haskins 1979; Dargaei et al., 2014). The separation between application and recording site was intended to assure that the response started in one region and then spread to the rest of the cluster. In essence, this mimics the intact nervous system, where depolarization is thought to expand out from a subset of neurons receiving cholinergic synaptic input (Brown and Mayeri, 1989; Dargaei et al., 2015).

When five clusters from other animals were treated with 100-μM genistein for 20 min prior to the experiment, the acetylcholine pressure-ejection consistently failed to elicit an afterdischarge-like response (Fig. 7D, *left*). Rather, the outcome ranged from small depolarization to small hyperpolarization to essentially



no effect. None of the neurons from the genistein-treated clusters fired action potentials when acetylcholine was delivered to the opposite side. Despite this, all the recorded cells were still fully excitable, as injecting depolarizing current after the acetylcholine application provoked spikes that clearly overshot 0 mV (Fig. 7D, *right*). When comparing simply the acetylcholine-induced membrane potential change of neurons from control ($n = 7$) and genistein-treated ($n = 5$) clusters the difference was significant (Fig. 7E).

DISCUSSION

Our earlier work provided strong evidence that acetylcholine is the input transmitter which starts the bag cell neuron afterdischarge. Applying acetylcholine to clusters in the abdominal ganglion causes an afterdischarge followed by a refractory period; moreover, synaptically evoked afterdischarges in the intact cluster are prevented by the nicotinic anatagonists, α-conotoxin ImI and mecamlyamine (White and Magoski, 2012). These anatagonists also inhibit the action of exogenously applied acetylcholine on voltage-clamped cultured bag cell neurons, specifically by blocking the ionotropic cholinergic receptor (White and Magoski, 2012; White et al., 2014). We now show that the acetylcholine-induced current is regulated by tyrosine phosphorylation, but not intracellular Ca^{2+} or PKC.

At the onset of the afterdischarge, there is a rise in cytosolic Ca^{2+}, followed by activation of the PKC pathway, and as the burst proceeds the phosphotyrosine levels decline (Fisher and Kaczmarek, 1990; Wilson and Kaczmarek, 1993). Ca^{2+} elevation in bag cell neurons is due to action potentials opening voltage-dependent Ca^{2+} channels (Knox et al., 1992; Fisher et al., 1994; Michel and Wayne, 2002; Geiger and Magoski, 2008). Gating of the ionotropic acetylcholine receptor causes sufficient depolarization to bring about action potentials and Ca^{2+} influx (the present study; White and Magoski, 2012; White et al., 2014). However, the link between the cholinergic receptor and PKC activation or tyrosine dephosphorylation is unknown. We previously found no indication that the receptor has a metabotropic signaling component (White and Magoski, 2012); thus, Ca^{2+} elevation or the depolarization itself may act on PKC and/or phosphotyrosine pathways. In PC12 cells, ionotropic acetylcholine receptor-induced Ca^{2+} entry causes both the translocation of PKC to the membrane and switching on of proline-rich tyrosine kinase 2 (Messing et al., 1989; Lev et al., 1995). In bag cell neurons, it is well established that PKC is activated early in the slow-phase, *ie*, by 1–2 min (Fink et al., 1988; Conn et al., 1989; Wayne et al., 1999). Likewise, the slow-phase sees tyrosine dephosphorylation regulating a non-selective cation channel (see end of *Discussion* for details) (Wilson and Kaczmarek, 1993; Magoski and Kaczmarek, 2005).

The Ca^{2+} influx following a 1-min depolarizing train-stimulus does not modulate the bag cell neuron acetylcholine-induced current. This is in contrast to Slater et al. (1985), who found that cholinergic currents in *Aplysia* RB cluster neurons were shortened by a calmodulin antagonist, implying that intracellular Ca^{2+} potentiates the response. That aside, ionotropic acetylcholine receptors in hippocampal and habenula neurons, chromaffin cells, or heterologous expression systems, are inhibited or show greater desensitization with elevated cytosolic Ca^{2+} (Khiroug et al., 1997, 1998, 2003; Fenster et al., 1999a; Guo and Lester, 2007). For bag cell neurons, a lack of negative regulation at the start of the afterdischarge may be advantageous, given that Ca^{2+}-dependent inhibition of the receptor would reduce the initial depolarization. The present finding, along with our prior report that the acetylcholine-induced current neither passes Ca^{2+} nor is modified by changes to extracellular Ca^{2+}, suggests Ca^{2+} does not control this channel (White et al., 2014).

The bag cell neuron acetylcholine-induced current is also not altered by turning on PKC with the phorbol ester, PMA. However, ionotropic acetylcholine receptors from *Torpedo* electric organ have been shown to be directly phosphorylated by PKC (Miles and Huganir, 1988; Swope et al., 1995). Furthermore, PKC accelerates recovery from desensitization of cholinergic receptors in chromaffin cells and α4β2 nicotinic receptors expressed in oocytes (Khiroug et al.,1998; Fenster et al., 1999a). Alternatively, PKC inhibits cholinergic receptors or intensifies

Fig. 5. Tyrosine kinase inhibition has minimal impact on action potential threshold and membrane current. (A) *Right panel*, sharp-electrode current-clamp recording of a cultured bag cell neuron initially set to −60 mV with continuous bias current. In the presence of DMSO, injecting a 50-ms, 1-nA depolarizing current (at *bar*) generates an action potential. *Left panel*, Summary graph indicating the average action potential threshold current, determined by injecting a 0.2–2nA (see *Results* for details), is not significantly different when comparing before (control) and 10 min after adding 100-μM genistein ($t_5 = 2.087$, $p > 0.05$, paired Student's *t*-test). (B) Group data for input resistance taken from the same neurons as *panel A* reveal no significant difference between before (control) and 10 min in genistein ($^{*}Z = -9.000$, $p > 0.05$, Wilcoxon matched-pairs signed rank test). (C) Whole-cell voltage-clamp recordings of cultured bag cell neurons held at −60 mV show no change in holding current following bath application (at *bar*) of DMSO (*upper*) or 25-μM PP1 (*middle*), while 100-μM genistein (*lower*) induces an inward current. Scale bars apply to all traces. (D) Average data indicating that the change in holding current induced by genistein differs significantly from that provoked by DMSO or PP1 ($F_{2,26} = 6.812$, $p \leq 0.005$ ordinary ANOVA, $p > 0.05$ for DMSO *vs* PP1, $^{*}p \leq 0.05$ for genistein *vs* DMSO or PP1, Bonferroni multiple comparisons test). (E) Voltage-gated Ca^{2+} currents evoked with 200-ms, 10-mV incremental square pulses from −60 to +40 mV in neurons voltage-clamped in Ca^{2+}-Cs^{+}-TEA-ASW using a Cs^{+}-based internal solution. Regardless of being treated with DMSO (*upper*) or 25-μM PP1 (*lower*) for ~20 min prior to recording, the currents in the two conditions are very much alike. Scale bars apply to both sets of traces. Currents subject to on-line leak subtraction (see *Experimental procedures*, *Whole-cell voltage-clamp recording from cultured bag cell neurons* for details). (F) Summary current–voltage relationships for Ca^{2+} current from cells in DMSO (*open circles*) or PP1 (*closed circles*). Peak current at each voltage is normalized to whole-cell capacitance and reported as current density. Between the two data sets, there is no significant difference in the Ca^{2+} current at those voltages where there is appreciable current (*ie*, −10 mV $t_{10} = 2.030172$, $p > 0.05$; 0 mV $t_{10} = 1.370$, $p > 0.05$; +10 mV $t_{10} = 1.449$, $p > 0.05$; +20 mV $t_{10} = 0.8606$, $p > 0.05$; +30 mV $t_{10} = 0.4322$, $p > 0.05$; +40 mV $t_{10} = 0.2456$, $p > 0.05$; +50 mV $t_{10} = 0.1686$, $p > 0.05$; all unpaired Student's *t*-test).

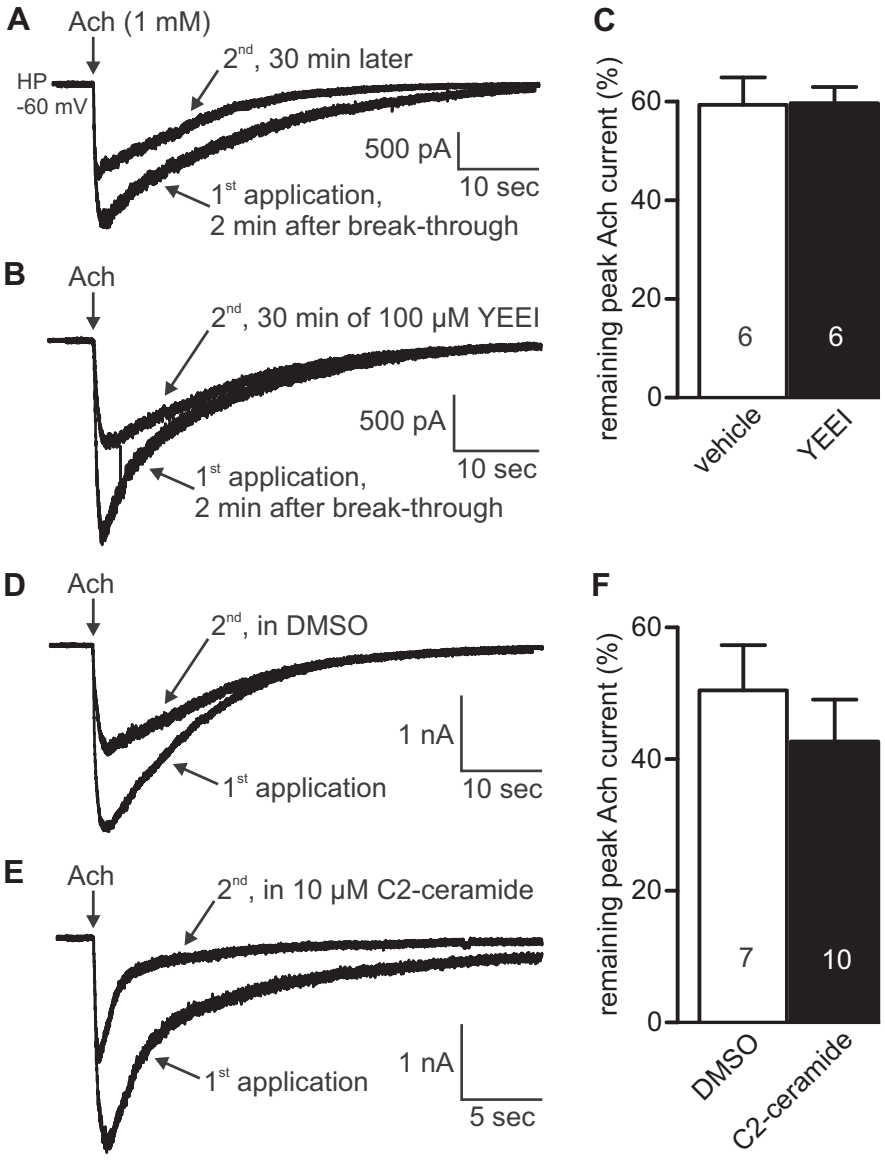


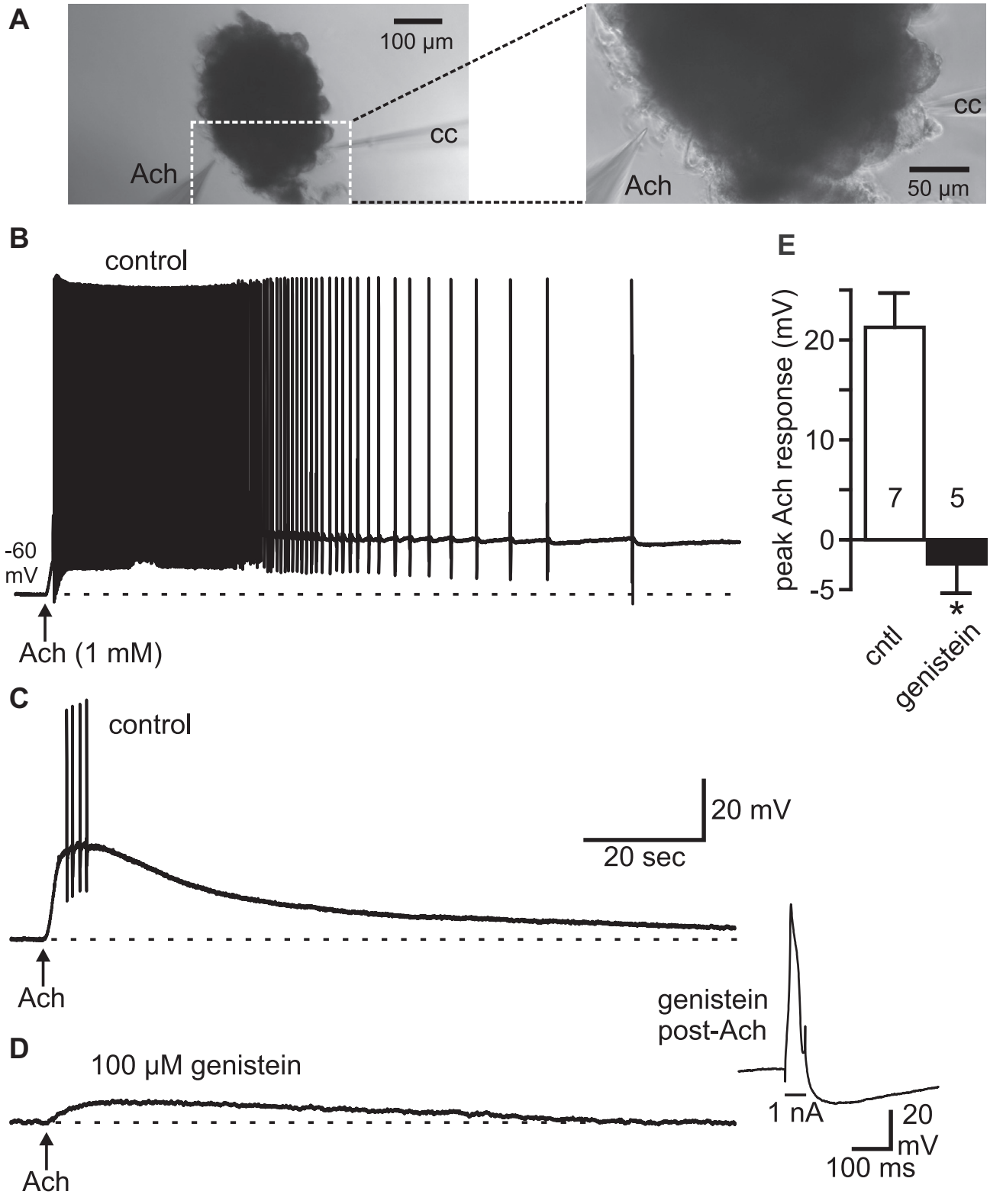
Fig. 6. The acetylcholine-induced current is not altered by increasing tyrosine phosphorylation. (A) A cultured bag cell neuron is voltage-clamped at −60 mV. Just after breaking through to gain whole-cell access, a cholinergic response is elicited with a 2-s pressure-ejection of 1-mM acetylcholine (Ach, at *arrow*). Following 30 min of intracellular perfusion with standard solution, a second application of acetylcholine produces a current that is around two-thirds of the first. Traces overlaid for clarity. (B) As per *panel A*, immediately after attaining whole-cell configuration, a cholinergic current is evoked by pressure-ejecting acetylcholine. Subsequent to a 30-min intracellular perfusion of standard solution supplemented with 100-μM of the src activator, YEEI peptide, delivering acetylcholine for a second time yields a current that is about half as large as the initial response. (C) For intracellular perfusion with the vehicle vs YEEI peptide, summary data reveal no significant difference in the remaining peak acetylcholine-induced current from the first to second application, with both conditions at ~60% (t_{10} = 0.05212, $p > 0.05$, unpaired Student's *t*-test). (D) After the first pressure-ejection of acetylcholine, DMSO is added to the bath and acetylcholine delivered a second time 20 min later. The second cholinergic current is nearly two-thirds as large as the first. (E) Bath applying 10-μM of the tyrosine kinase activator, C2-ceramide for 20 min leads to a similar reduction of the second acetylcholine-induced current compared to the initial response. (F) Evaluating the average remaining peak current in DMSO and C2-ceramide shows any difference does not reach the level of significance (t_{15} = 0.7845, $p > 0.05$, unpaired Student's *t*-test).

desensitization in sympathetic ganglion neurons as well as *Aplysia* R15 or RB-group neurons (Downing and Role, 1987; Papp and Hoyer, 1996). For bag cell neurons, the activation of PKC with the slow-phase leads to an enhancement of both voltage-gated Ca^{2+} current (which increases action potential height) and non-selective cation current (which maintains the depolarization) (DeRiemer et al., 1985a,b; Wilson et al., 1998; Magoski et al., 2002; Tam et al., 2009). These events occur after acetylcholine has provided the initial input, so a lack of PKC-dependent regulation of the cholinergic receptor may have little physiological implication.

Inhibition of tyrosine kinases attenuates the bag cell neuron acetylcholine-induced current. This is apparent in the small amount of relative current after either PP1 (25% remaining) or genistein (10% remaining). These data are consistent with the cholinergic current being upregulated by tyrosine phosphorylation, *ie*, blocking tyrosine kinases allows tyrosine phosphatases to exert more influence, which lowers phosphotyrosine levels and reduces the acetylcholine response. Comparable effects of genistein, PP1, and other similar-acting reagents have been reported for ionotropic acetylcholine-induced currents in submucosal or pelvic ganglion neurons and chromaffin cells, as well as cell lines expressing α3β4α5 nicotinic receptors (Glushakov et al., 1999; Wang et al., 2004; Kim et al., 2011). For the latter, dephosphorylation does not hasten removal of the channel from the membrane, suggesting the effect is on receptor function rather than number (Wang et al., 2004). There are also contrary reports, such as tyrosine kinase inhibition causing an enhancement of the α7 nicotinic receptor current endogenous to hippocampal neurons and SH cells, or expressed in oocytes (Charpantier et al., 2005). It is unknown whether the bag cell neuron acetylcholine receptor is basally tyrosine phosphorylated, and therefore presumably dephosphorylated in the presence of genistein or PP1. However, both the endogenous receptor in chromaffin cells and α3 or β4 nicotinic receptor subunits in cell lines are tyrosine phosphorylated at rest (Wang et al., 2004). Our effort to turn on tyrosine kinases, using either the src activator, YEEI peptide, or the tyrosine kinase activator, C2-ceramide, does not enhance the acetylcholine-induced current. This may indicate that the receptor is tyrosine phosphorylated

at rest. Although such an interpretation should be guarded, given that the literature provides no report of a biochemical assay showing either YEEI peptide or C2-ceramide directly alters tyrosine phosphorylation levels in *Aplysia* nervous tissue.

In keeping with our previous work (White and Magoski, 2012; White et al., 2014), the present study confirms that pressure-applying acetylcholine to cultured bag cell neurons results in depolarization and spiking. We now show that delivering acetylcholine to one side of an



isolated, de-sheathed bag cell neuron cluster, while recording from a neuron on the opposite side, can elicit afterdischarge-like bursts. However, both *in vitro* and *ex vivo*, treatment with genistein lessens the cholinergic depolarization (in the case of the cluster it is largely absent) and abolishes action potential firing. Given that spike threshold, input resistance, and voltage-gated Ca^{2+} current (which carries the action potential upstroke in these neurons; DeRiemer et al., 1985b) do not change under tyrosine kinase inhibition, it is unlikely that a drop in excitability accounts for the lack of spiking to acetylcholine in genistein. In fact, a reasonable prediction would be a slightly stronger depolarization in the presence of genistein, on account of this reagent producing a small inward current (the nature of which is unknown). That stated, all current-clamp measurements were made starting at −60 mV, which was set with bias current before every step-current injection or acetylcholine pressure-ejection. Thus, if the genistein-evoked current did produce a minor depolarization, its influence on the cholinergic response would have been mitigated. The more probable reason for the smaller depolarization and an absence of firing to acetylcholine is that genistein markedly decreases the acetylcholine-induced current.

The impact of genistein on the cholinergic depolarization in the isolated cluster is more profound than in culture. Beyond the obvious difference of the cluster containing many neurons, it also has the distinction of wide-spread electrical coupling (Blankenship and Haskins, 1979; Dargaei et al., 2014). Presumably, pressure-applied acetylcholine provokes depolarization in one, or quite possibly several, neuron (s) on one side of the cluster, and this voltage change propagates through the cluster to the recording site on the other side. When genistein lessens the initial depolarization, the recipient neuron(s) may simply not reach threshold, much like single cultured neurons. Alternatively, the current sink formed by the coupled cells could limit electrotonic spread, such that the now smaller depolarization is insufficient to recruit a neuron which is remote from the cholinergic input. Of course, we cannot exclude the possibility that the drop in phosphotyrosine levels also impacts gap junctions directly; if the innexins are inhibited by genistein, then voltage propagation would be restricted.

Tyrosine phosphorylation also regulates a voltage-dependent cation channel that supplies ongoing depolarizing drive for the afterdischarge. In particular, tyrosine dephosphorylation during the slow-phase promotes the physical association of the cation channel with PKC, which is a positive gating factor for this current (Wilson and Kaczmarek, 1993; Wilson et al., 1998; Magoski et al., 2002; Magoski and Kaczmarek, 2005). The channel-PKC association can also be encouraged by exposure to inhibitors like PP1 (Magoski and Kaczmarek, 2005). However, conditions boosting tyrosine phosphorylation, such as adding src kinase, serve to decrease cation channel opening and lower the frequency of the channel-PKC association (Magoski and Kaczmarek, 2005). Thus, tyrosine dephosphorylation later in the afterdischarge would be expected to elevate the cation current while suppressing the acetylcholine-induced current.

Along with cation channel activation, the slow-phase also sees the bulk of egg-laying hormone secretion during the ∼1-Hz firing (Loechner et al., 1990). Downregulating the acetylcholine receptor may prevent inappropriate excitatory input from changing the spike rate or membrane potential, thereby protecting those circumstances which ostensibly favor peptide release. Furthermore, our earlier work found that if an intact bag cell neuron cluster was rendered refractory by undergoing a synaptically elicited afterdischarge, the depolarizing response to a subsequent application of exogenous acetylcholine is practically eliminated (White and Magoski, 2012). Given the effect of genistein on acetylcholine-stimulated bursting in isolated clusters, a diminished acetylcholine-induced current may explain this reduced cholinergic response during the refractory period. Overall, by influencing multiple conductances, the phosphotryosine level has the capacity to control and/or maintain different states of bag cell neuron excitability necessary for reproduction.

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Fig. 7. Acetylcholine initiates an afterdischarge-like response in isolated clusters and this is prevented by tyrosine kinase inhibition. (A) *Left panel*, view of a de-sheathed bag cell neuron cluster in tcASW indicating the placement of the acetylcholine (Ach)-containing pressure-ejection electrode and the intracellular current-clamp (cc) recording sharp-electrode on the opposite side of the cluster. *Right panel*, magnified view of region in dashed-box focusing on the ejection pipette (*lower-left*) and sharp-electrode (*middle-right*). All acetylcholine applications and intracellular recordings involving the isolated cluster were carried out in this fashion. (B) A 2-s pressure-ejection of 1-mM acetylcholine (Ach, at *arrow*) delivered to one side of a cluster initiates an afterdischarge in an individual bag cell neuron recorded at least 200 μm away from the application site (recording truncated at ∼2.5 min). The membrane potential of the neuron is set to −60 mV with bias current prior to delivery of acetylcholine. Typical of four out of seven control clusters. (C) In a separate cluster from a different animal, the same type of 2-s acetylcholine application causes a bag cell neuron to depolarize by ∼30 mV, which is accompanied by a short burst of action potentials. However, instead of transitioning to prolonged firing, the cell repolarizes toward resting potential. Representative of three out of seven control clusters. Calibration bars apply to *panels B*, *C*, and *D*, *right*. (D) *Left panel*, in another cluster from a separate animal, treated for 20 min with 100 μM genistein, pressure-ejecting acetylcholine evokes a modest, 7 mV depolarization with no action potential firing that gradually recovers to −60 mV. *Right panel*, subsequent to the application of acetylcholine, injection of a 1-nA depolarizing current (at *bar*) shows the neuron fully capable of generating an action potential in the presence of genistein. Characteristic of all five genistein experiments. Calibration bars apply only to *right panel*. (E) Summary data of the acetylcholine-induced response for untreated (control) clusters and clusters given 100-μM genistein. Compared to the control depolarization of ∼20 mV, genistein renders the response as an ∼2 mV hyperpolarization ($^{*}t_{10} = 4.991$, $p \leq 0.001$, unpaired Student's *t*-test).

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