Nicotine inhibits potassium currents in Aplysia bag cell neurons

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A PRIMARY DETERMINANT OF NEURONAL excitability is both the influence of leak K+ channels on the resting membrane potential (Mathie et al. 2010) and the ability of voltage-gated K+ channels to alter responsiveness or action potential dynamics (Johnston et al. 2010). Examples of this include serotonin acting through 5-HT1A receptors to close S-type K+ channels in Aplysia sensory neurons (Lee et al. 2009; Shuster et al. 2006; White and Magoski 2012). Acetylcholine generates an inward current through opening of a nonselective cation channel (White and Magoski 2012). Acetylcholine generates an inward current through opening of a nonselective cation channel (White and Magoski 2012). Acetylcholine generates an inward current through opening of a nonselective cation channel (White and Magoski 2012). Acetylcholine generates an inward current through opening of a nonselective cation channel (White and Magoski 2012).
MATERIALS AND METHODS

Animals and cell culture. Adult *A. californica* weighing 150–500 g were obtained from Marinus (Long Beach, CA), housed in an ~300-liter aquarium containing continuously circulating aerated artificial sea water (Instant Ocean; Aquarium Systems, Mentor, OH) at 15°C on a 12:12-h light-dark cycle, and fed Romaine lettuce 5 times per week. All experiments were approved by the Queen's University Animal Care Committee (protocols Magoski-2009-065 or Magoski-2013-041).

For primary cultures of isolated bag cell neurons, animals were anesthetized by an injection of isotonic MgCl2 (~50% body wt), and the abdominal ganglion was removed and treated with neutral protease (13.33 mg/ml; 165859; Roche Diagnostics, Indianapolis, IN) dissolved in tissue culture artificial sea water (tcASW; composition in mM: 460 NaCl, 10.4 KCl, 11 CaCl2, 55 MgCl2, 15 HEPES, 1 mg/ml glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin; pH 7.8 with NaOH) for 18 h at 20–22°C. The ganglion was then transferred to fresh tcASW for 1 h, after which the bag cell neuron clusters were dissected from their surrounding connective tissue, and with the use of a fire-polished Pasteur pipette for gentle trituration, neurons were dispersed in tcASW onto 35-× 10-mm polystyrene tissue culture dishes (430165; Corning, Corning, NY; or 353001; Falcon Becton-Dickinson, Franklin Lakes, NJ). Cultures were maintained in tcASW in a 14°C incubator and used for voltage- or current-clamp experiments (see below) within 1–3 days. Data were collected at room temperature (20–22°C). Salts were obtained from Fisher Scientific (Ottawa, ON, Canada), ICN (Aurora, OH), or Sigma-Aldrich (St. Louis, MO).

Whole-cell voltage-clamp recording. Voltage-clamp recordings were made using an EPC-8 amplifier (HEKA Electronics; Mahone Bay, NS, Canada) and the tight-seal whole cell method. Microelectrodes were pulled from 1.5-mm external, 1.2-mm internal diameter borosilicate glass capillaries (TW150F-4; World Precision Instruments, Sarasota, FL) and had a resistance of 1–2 MΩ when fire-polished and filled with intracellular saline (see below). Before seal formation, pipette junction potentials were nulled. After seal formation, the pipette capacitive current was canceled, and after breakthrough, the whole cell capacitive current was canceled, while the series resistance (3–5 MΩ) was compensated to 80% and monitored throughout the experiment. Data were acquired using the Clampex program of the pCLAMP suite (v10.0; Molecular Devices, Sunnyvale, CA), a Digidata 1322A analog-to-digital converter (Molecular Devices), and an IBM-compatible personal computer. Current was filtered at 1 kHz with the EPC-8 Bessel filter and sampled at 2 kHz.

Initial recordings were made in normal artificial seawater (nASW; composition as per tcASW but lacking glucose and antibiotics) with standard intracellular saline [composition in mM: 500 KCl,-apastarte, 70 KCl, 1.25 MgCl2, 10 HEPES, 11 glucose, 10 glutathione, 5 EGTA, 5 adenosine 5′-triphosphate disodium salt hydrate (A3377; Sigma-Aldrich), and 0.1 guanosine 5′-triphosphate sodium salt hydrate (GTP; G8877; Sigma-Aldrich)]; pH 7.3 with KOH in the recording pipette. The free intracellular Ca2+ concentration was set at 300 nM by adding 3.75 mM of CaCl2, as calculated by WebMaxC (http://www.stanford.edu/~capan/webmaxc.htm). In some instances, K+ was replaced with Cs+ or GTP replaced with 10 mM guanosine 5′-(β-thio)diphosphate trilithium salt (GDPβS; G7637; Sigma-Aldrich). For isolation of K+ currents, Na+−free/Ca2+-free ASW was used as the external solution, with Na+ replaced by N-methyl-D-glucamine (NMDG·) and Ca2+ replaced by Mg2+. As necessary, online leak subtraction employed a P/4 protocol with subpulses of opposite polarity and one-quarter the magnitude of each test pulse (Bezanilla and Armstrong 1977). The inter-subpulse interval was 500 ms, with 5–10 s between test pulses. Junction potentials of 15 and 23 mV were calculated for the intracellular saline vs. nASW and Na+−

Sharp-electrode current-clamp recording. Current-clamp recordings were made in nASW using an AxoClamp 2B amplifier (Molecu-

ular Devices) and the sharp-electrode bridge-balanced method. Microelectrodes were pulled from 1.2-mm external, 0.9-mm internal diameter borosilicate glass capillaries (TW120F-4; World Precision Instruments) and had a resistance of 5–20 MΩ when filled with 2 M K+-acetate, 10 mM HEPES, and 100 mM KCl (pH 7.3 with KOH). When necessary, neurons were set to ~60 mV using the AxoClamp DC current command while Clampex was used to inject step current. Voltage was filtered at 3 kHz, using the AxoClamp Bessel filter, and sampled at 2 kHz as in Whole cell voltage-clamp recording.

Drug application and reagents. The culture dish served as the bath. For single-cell microperfusion, i.e., superfusion, a micromanipulator-controlled square-barrel glass pipette (~500-μm bore) was 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 (0.5–1 ml/min) of control extracellular saline over the neuron, which was switched to saline containing nicotine for a specific amount of time by activating the appropriate stopcock. Nicotine was also pressure-ejected from an unpolished patch pipette (1- to 2-μm bore) for 2 s at 75–150 kPa using a PMI-100 pressure microinjector (Dagan, Minneapolis, MN). To access the pharmacology of the nicotine-evoked response, antagonists were delivered directly to the bath between successive pressure applications, separated by ~10 min. As previously undertaken with bag cell neurons by Fisher et al. (1993) and ourselves (White and Magoski 2012), perfusion was typically not employed during pressure application; however, the pipette was removed from the bath immediately after each ejection to minimize leakage and possible desensitization. In some experiments, nASW, nicotine, or the structurally similar agonist, epibatidine (Badio and Daly 1994), was continuously pressure-applied for 10 s.

Drugs were made up as stock solutions in water, frozen at ~20°C, and then diluted down to a working concentration in the extracellular or intracellular solutions as needed: nicotine (N0257; Sigma-Aldrich), mecamylamine hydrochloride (M9020; Sigma-Aldrich), α-conotoxin ImI (3119; Tocris Bioscience), hexamethonium dichloride (H2138; Sigma-Aldrich), (±)-epibatidine dihydrochloride hydrate (E1145; Sigma-Aldrich).

Analysis. The Clampfit program of pCLAMP was used to determine the amplitude and time course of changes in ligand- or voltage-gated current as well as the membrane potential or action potentials. For changes to steady-state current or membrane potential, after 1 min of baseline, two cursors were placed immediately before the current or voltage change while an additional two cursors were positioned to encompass the peak response. Clampfit then calculated the average current or voltage between the cursor pairs. The maximal response was taken as the difference between the average baseline and peak values. Similar measurements were made for voltage-dependent current, which in most cases was then normalized to cell size by dividing by the whole cell capacitance (as determined by the EPC-8 slow capacitance compensation circuitry). The peak and time to peak of the action potential were determined using Clampfit by setting cursors immediately before the spike and along the falling phase. The action potential half-width was found by taking the time from the peak to halfway down the falling phase between the peak and the trough. Conductance was derived using Ohm’s law (G = IV) from the current during a 1-s step from 0 to ~10 mV. In cases where nicotine was applied twice, the subsequent application occurred after an ~10-min interval, and any change in peak current was quantified by expressing the second response as a percentage of the first. For display, some nicotine-evoked current traces were filtered off-line to between 20 and 80 Hz using the Clampfit Gaussian filter; comparison with original data ensured that this second filtering brought about no change in amplitude or kinetics. The concentration-response curve was fitted with a Hill slope equation:

\[
Y = bottom + \left( top - bottom \right) \cdot \left[ 1 + 10^{\left( logEC50 - X \right) \cdot \text{Hill slope}} \right]
\]

using Prism (v5.04; GraphPad Software, La Jolla, CA) and the least-squares method, where bottom is the basal response, top is the
maximal response, and EC_{50} is the 50% effective concentration. The fast and slow time constants (τ_f and τ_s) of the delayed-rectifier K^+ currents were determined by fitting tail currents with a standard two-term exponential:

\[
f(t) = \sum_{i=1}^{n} A_i e^{-t/\tau_i} + C
\]

using Clampfit and the Chebyshev method, where \(i\) is the index of summation, \(n\) is the upper bound of summation, \(A\) is the amplitude, \(\tau\) is the time constant, and \(C\) is the constant y-offset. The same equation was also used to fit delayed-rectifier K^+ current use-dependent inactivation plots.

Statistical analysis was performed using Prism. The Kolmogorov-Smirnov method was used to test data sets for normality. To test whether the mean differed between two groups, either Student's paired or unpaired t-test (for normally distributed data) with the Welch correction as necessary (for unequal standard deviations) or the Mann-Whitney U-test (for not normally distributed data) was used. Comparing more than two means involved a one-way ordinary or repeated-measures analysis of variance (ANOVA), followed by the Dunnett multiple comparisons test. Means were considered significantly different if the two-tailed P value was <0.05. Data are means ± SE.

RESULTS

While characterizing the more orthodox, linear inward current induced by nicotine at −60 mV (see White et al. 2014 for details), we noticed an apparent voltage-dependent current, which began at approximately −30 mV and then increased in magnitude with further depolarization. This was similar to the inverted U-shaped I/V relationship caused by nicotine in dorsal unpaired median cells of the cockroach, Periplanta (Courjaret and Laped 2001; Thany et al. 2008). That result was attributed to an atypical nicotinic receptor that closed upon agonist binding. Thus we sought to determine if the nicotine-evoked current at depolarized potentials was receptor mediated or, as reported in rat heart (Wang et al. 2000a, 2000b), due to nicotine in some way blocking K^+ channels.

Nicotine produces negative slope in the I/V relationship of bag cell neurons. Cultured bag cell neurons were whole cell voltage-clamped in nASW and perfused intracellularly for at least 5 min with our standard K^+ -aspartate-based intracellular saline. A total of four 10-s ramps from −90 to +20 mV (inset) were given, each separated by 2 min. In control conditions (black trace), before the addition of nicotine, subtracting the first ramp current from the second furnished an essentially flat I/V relationship, with only a small outward current at positive voltages. However, subtracting the third ramp current from the fourth [taken after the neuron was exposed to 3 mM nicotine (Nic) for the full 2 min (gray trace)] revealed an inward current characteristic of negative slope in the I/V relationship (i.e., developing at approximately −30 mV and peaking around 0 mV). Between the second and fourth ramp, 3 mM nicotine was continuously perfused over the same neuron, i.e., 2 min of nicotine. On the basis of our previous work, we know that this lengthy nicotine exposure would result in desensitization of any nicotinic ionotropic receptors that normally pass inward current at −60 mV (White et al. 2014). The difference current obtained by subtracting the current produced by the third ramp from that triggered by the fourth ramp (in nicotine) yielded a negative-slope I/V relationship that was inward and voltage dependent with an onset near −30 mV (Fig. 1A, gray trace).

The subtraction current in nicotine reached maximal amplitude between −10 and 0 mV. On average, this corresponded to a peak of approximately −450 pA, which was significantly different from the approximately +50-pA current elicited by the ramp under control conditions (n = 8; Fig. 1B). With respect to total membrane current, Fig. 1C provides a nonsubtracted current trace, produced by the ramp, from one of the eight nicotine-exposed neurons. In this case, a negative slope was apparent in the total I/V relationship beginning at −10 mV, with a peak inward current around 0 mV.

A concentration-dependent, nicotine-evoked current at depolarized potentials. Our earlier study showed that nicotine induced an ionotropic inward current at −60 mV, which reversed at approximately −25 mV, but passed little outward current at voltages more positive than the reversal potential (White et al. 2014). Since this reversal potential overlaps with the development of the voltage-dependent current at approximately −30 mV, cultured bag cell neurons were whole cell
voltage-clamped to $-10 \text{ mV}$, to isolate as much as possible the second type of nicotine-evoked response. To generate a concentration-response relationship, nicotine was superfused at concentrations ranging from 300 $\mu$M to 30 mM, for 30 s each, with 10 min between applications. Responses were repeatable, and up to three concentrations were delivered per neuron. Example traces during perfusion of 300 $\mu$M, 1 mM, 3 mM, or 10 mM nicotine are shown in Fig. 2A. Average current densities (peak current normalized to cell capacitance) from multiple concentrations yielded a curve with a peak of $-600 \text{ pA/pF}$, a Hill slope of 1.08, indicating lack of cooperative binding, and an EC$_{50}$ of 1.38 mM (Fig. 2B). Based on these values, it appears that the current at $-10 \text{ mV}$ does not involve the same receptor as the current induced by nicotine at $-60 \text{ mV}$, which was cooperative (Hill slope = 2.4) and left-shifted (EC$_{50}$ = 240 $\mu$M) (White et al. 2014).

To test for a change in conductance during nicotine exposure, neurons were held at 0 mV and a 1-s step to $-10 \text{ mV}$ was applied ($n = 9$; Fig. 2C, bottom). Before the introduction of nicotine, under control conditions, the hyperpolarizing step produced an inward current of usually several hundred picamperes (Fig. 2C, top, black trace). Delivering the pulse 2 min later, during the current induced by 3 mM nicotine (a concentration near the EC$_{50}$), resulted in a noticeably smaller step current (Fig. 2C, top, gray trace). On average, compared with the control conductance of $\sim 35 \text{ nS}$, the conductance in the presence of nicotine was significantly smaller at $\sim 27 \text{ nS}$ (Fig. 2D). This suggested that the nicotine-evoked response at $-10 \text{ mV}$ was due to a channel closure, as opposed to our preceding work demonstrating channel opening by nicotine at $-60 \text{ mV}$ (White et al. 2014).

The nicotine-evoked response at $-10 \text{ mV}$ is dependent on $K^+$. An initial examination of the response to nicotine at holding potentials ranging from $-10$ to $+60 \text{ mV}$ revealed that the current increased in magnitude with more positive voltages (data not shown). The augmentation of inward current at more depolarized potentials was similar to the decrease-conductance $K^+$-dependent postsynaptic potential in Aplysia L14 ink motor neurons (Carew and Kandel 1977). Thus the response in cultured bag cell neurons may involve $K^+$ channel block, and we attempted to occlude this by replacing the $K^+$ in the whole cell pipette with Cs$^+$, a $K^+$ channel blocker (Adelman and Senft 1966; Hille 2001). During holding at $-10 \text{ mV}$, a 1-min continuous perfusion of 3 mM nicotine led to a relatively large nongates current with $K^+$-based internal solution ($n = 10$; Fig. 3A, top); however, inclusion of Cs$^+$ in the pipette ($n = 12$) either decreased or eliminated the response entirely (Fig. 3A, bottom). The mean peak current density was $\sim 0.1 \text{ pA/pF}$ following intracellular Cs$^+$ perfusion, which was significantly different from the $\sim 0.7 \text{ pA/pF}$ with intracellular $K^+$ perfusion (Fig. 3B).

The nicotine-evoked response at $-10 \text{ mV}$ is not dependent on G proteins. There is a possibility that the nicotine response is due to a nicotinic receptor metabotropically closing ion channels. For example, Tieman et al. (2001) used both electrophysiology and chromatography to show that nicotine-gated ionotropic receptors can activate lipoxigenase via G proteins in neural tissue and identified neurons from Aplysia. As such,
cultured bag cell neurons were perfused intracellularly for 30 min with standard internal solution plus 10 mM GDPβS, a nonhydrolyzable GDP analog that inhibits heterotrimetric G proteins by competing with GTP for α-subunit binding (Eckstein et al. 1979). Compared with controls perfused intracellularly with GTP (n = 6; Fig. 3C, top), introducing GDPβS did not alter the response to 3 mM nicotine at −10 mV (n = 5; Fig. 3C, bottom). In these experiments, we employed a 2-s pressure application of nicotine, which we found as effective as perfusion in bringing about an effect. The summary data revealed no significant difference in the current density for GDPβS vs. GTP, with both at −0.3 pA/pF (Fig. 3D). We previously showed that GDPβS eliminated an outward current in bag cell neurons induced by FMRFamide (White and Magoski 2012), which is known to act via metabotropic receptors in molluscs (Brezina et al. 1987; Piomelli et al. 1987a, 1987b). Additional work in our laboratory and elsewhere finds GDPβS to effectively prevent G protein-mediated currents or depolarizations in neurons from both Aplysia and the pulmonate mollusc, Lymnaea (Bolshakov et al. 1993; Kehoe 1994; Lemos and Levitan 1984; Magoski et al. 1995; Tam et al. 2011).

Pharmacology of the nicotine-evoked response at −10 mV. We next explored the prospect that the nicotine-evoked response at −10 mV involved a traditional nicotinic receptor. Nicotine was pressure-applied twice to whole cell voltage-clamped cultured bag cell neurons at −10 mV; applications were separated by a minimum of 10 min. Classical nicotinic antagonists were introduced directly into the bath after the first nicotine application, and their relative effectiveness determined by calculating the percent change in the peak current between the first and second nicotine responses (see MATERIALS AND METHODS, Drug application and reagents and Analysis). Under control conditions, without the addition of any antagonist to the bath, the second ejection of nicotine at −10 mV produced a response that was essentially equal to the first (n = 10; Fig. 4A, top vs. bottom, and C). A concentration of 100 μM mecamylamine (Ascher et al. 1979; Stone et al. 1956), which blocks the nicotine current at −60 mV (White et al. 2014), failed to alter the nicotine-evoked response at −10 mV and resulted in a similar percentage of remaining current as that of control, i.e., ~100% (n = 5; Fig. 4B and C). Likewise, neither 1 μM α-conotoxin ImI (n = 7) (Ellison et al. 2003; McIntosh et al. 1994) nor 100 μM hexamethonium (n = 6) (Ascher et al. 1978; Kehoe 1972) altered the second nicotine-evoked response (Fig. 4C).

Short-term nicotine treatment inhibits voltage-gated K+ currents. Thus far, our observations suggested that nicotine inhibits some form of K+ current. We next examined whether a similar outcome occurs with the fast-inactivating A-type (I_A) and delayed-rectifier K+ (I_K) currents. To isolate K+ currents, we replaced the extracellular Na+ and Ca2+ with NMDG+ and Mg2+, respectively, while using our standard K+–aspartate-
based intracellular solution. The Na\(^+\)-free, Ca\(^{2+}\)-free ASW has been used by our laboratory and others to observe K\(^+\) currents in bag cell neurons (Dargaei et al. 2014; Zhang et al. 2002). To record \(I_A\), which requires hyperpolarization from resting potential to deinactivate, cultured neurons were voltage-clamped at a holding potential of \(-90\,\text{mV}\), followed by 500-ms voltage steps to \(-80\), \(-60\), \(-40\), and \(-20\,\text{mV}\) (Fig. 5A, bottom) (Strong 1984). \(I_K\) were studied by holding at \(-60\,\text{mV}\), which inactivates essentially all \(I_A\), and then making 200-ms pulses to \(-40\), \(-20\), 0, \(+20\), and \(+40\,\text{mV}\) (Fig. 5B, bottom) (Strong and Kaczmarek 1986). For both currents, before each step, a P/4 protocol was used to subtract leak currents (see MATERIALS AND METHODS, Whole cell voltage-clamp recording). When possible, the same neuron was used to examine \(I_A\) and \(I_K\), as well as tail currents for the isolation of the two components of \(I_K\) (see last paragraph of this section).

In control neurons (\(n = 11\)), \(I_A\) was first apparent during the 500-ms step to \(-60\,\text{mV}\) from \(-90\,\text{mV}\), but was rapidly and completely inactivated to baseline by the end of the pulse (Fig. 5A, top). Similarly, steps to \(-40\) and \(-20\,\text{mV}\) also activated the current, albeit they were larger (peak \(-30\,\text{pA/pF}\) at \(+20\,\text{mV}\)), had faster onset kinetics, and lacked complete inactivation over the duration of the stimulus. In separate neurons, where \(10\,\text{mM}\) nicotine was perfused for at least \(10\,\text{min}\) before recording (\(n = 11\)), both the peak and steady-state \(I_A\) were diminished (Fig. 5A, middle). When normalized to cell capacitance, the peak current density was significantly less in the presence of nicotine at both resting membrane potential (\(-60\,\text{mV}\)) and moderately depolarized voltages (\(-40\) and \(-20\,\text{mV}\)) compared with controls (Fig. 5C). The blocking effect of nicotine appeared voltage dependent, based on a fourfold reduction at \(-20\,\text{mV}\) compared with a twofold reduction at \(-40\,\text{mV}\).

A similar drop in peak current magnitude occurred with \(10\,\text{mM}\) nicotine exposure for \(I_K\). In control neurons (\(n = 11\)), from a holding potential of \(-60\,\text{mV}\), very little current was evident without substantial depolarization, i.e., only during the 200-ms step to \(-20\,\text{mV}\) did a relatively slowly activating K\(^+\) current initially appear (Fig. 5B, top). However, current activation and magnitude was progressively accelerated with step depolarizations beyond \(-20\,\text{mV}\) (peak \(-35\,\text{pA/pF}\) at \(+40\,\text{mV}\)). By the end of the pulse, much of the sustained current was still present. Nicotine (\(n = 12\)) attenuated both the peak and sustained responses (Fig. 5B, middle), with the \(I_K\) peak current density being significantly reduced at all voltages from \(-20\) to \(+40\,\text{mV}\) (Fig. 5D). Contrary to the effect of nicotine on \(I_A\), the block of \(I_K\) was not augmented by depolarization.

The actions of nicotine on both \(I_A\) and \(I_K\) were reversible. To demonstrate this, we compared each current before, during, and after \(30\,\text{s}\) of \(10\,\text{mM}\) nicotine perfusion (\(n = 7\)). Currents were evoked by holding neurons at specific voltages and stepping to only one potential (to \(-40\) from \(-90\,\text{mV}\) for \(I_A\) and to \(+30\) from \(-60\,\text{mV}\) for \(I_K\)). For \(I_K\), the 500-ms step to \(-40\,\text{mV}\) (Fig. 5E, bottom) produced a typical fast-activat-

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**Fig. 4.** Antagonist profile for the nicotine-evoked response at \(-10\,\text{mV}\). Shown are responses to \(10\,\text{s}\) of pressure-applied \(3\,\text{mM nicotine (Nic)}\) in cultured bag cell neurons under whole cell voltage-clamp at a \(-10\,\text{mV}\) holding potential (HP) in nASW with K\(^-\)-based internal solution. A, top: in control, the response evoked by the first nicotine application was very similar to that produced 10 min later by the second application (bottom). Because there was no desensitization, antagonists were evaluated by delivering drug during the intervening period and measuring any change between the initial and latter response. Scale bars apply to both traces. B: in a separate neuron, bath delivery of \(100\,\mu\text{M}\) mecamylamine, a nicotinic receptor blocker, did not alter the second response evoked by pressure-applied nicotine. Scale bars apply to both traces. C: summary data showing the percentage of remaining current between the second and first nicotine applications. None of the classical nicotinic receptor blockers, i.e., mecamylamine (mec), \(\alpha\)-conotoxin Iml (Iml), or hexamethonium (hex), significantly altered the magnitude of the nicotine-evoked response (ordinary ANOVA). Numbers in bars indicate number of neurons.
Nicotine led to a significant decrease in the average peak $I_A$ density by ~75%, which returned to control levels upon wash (4.20 ± 0.63 pA/pF in control vs. 1.00 ± 0.23 pA/pF in nicotine vs. 4.53 ± 0.66 pA/pF in wash; $n = 7$; $P < 0.0001$, repeated-measures ANOVA; $P < 0.05$, Dunnett multiple comparisons posttest). For $I_K$, a characteristic slowly inactivating current was induced with the 200-ms step to +30 mV (Fig. 5F, bottom). In a manner similar to that for $I_A$, nicotine perfusion clearly reduced $I_K$, which completely recovered during wash for 10 min (Fig. 5F, top). The mean peak $I_K$ density was significantly lowered by around two-thirds, but this was completely restored upon return to normal extracellular saline (31.56 ± 3.69 pA/pF in control vs. 11.76 ± 1.17 pA/pF in nicotine vs. 33.50 ± 4.21 pA/pF in wash; $n = 7$; $P < 0.0001$, repeated-measures ANOVA; $P < 0.05$, Dunnett multiple comparisons posttest).

Strong and Kaczmarek (1986) demonstrated that $I_K$ can be separated into two components, one fast and the other slow, by fitting the tail currents elicited upon return to negative potentials from strong depolarization. Moreover, based on the considerable variation in the ratio of the slow to the fast component, as well as the two components being differentially mod-
ulated by cAMP, they concluded that the components represented two distinct voltage-dependent delayed-rectifier \( K^+ \) currents. Using a protocol similar to theirs, we held neurons at \(-50 \text{ mV} \) and delivered five 100-ms steps to \(+30 \text{ mV} \) at 0.2 Hz, with the interstep interval incorporating a P4 leak subtraction protocol (Fig. 6A, bottom). The resulting currents were averaged, and the tail current following re polarization to \(-50 \text{ mV} \) was fit with a two-term exponential decay (\( \tau_{\text{fast}} \) representing the inactivating component and \( \tau_{\text{slow}} \) representing the sustained component of \( I_{\text{K}} \), respectively). A typical control current (\( n = 12 \)) is displayed in Fig. 6A, top, with the approximate locations of the two time constants. The average \( \tau_{\text{fast}} \) was \(-1 \text{ ms} \), whereas \( \tau_{\text{slow}} \) was \(-11 \text{ ms} \) (Fig. 6B). As expected, the peak current was lowered by about twofold when 10 mM nicotine (\( n = 13 \)) was delivered to the bath (31.3 \( \pm \) 1.2 pA/pF in control vs. 14.9 \( \pm \) 1.1 pA/pF in nicotine; \( P < 0.0001 \), unpaired Student’s \( t \)-test; Fig. 6A, middle). However, nicotine inhibited both components of \( I_{\text{K}} \), because neither \( \tau_{\text{fast}} \) nor \( \tau_{\text{slow}} \) were changed and the summary data showed the time constants to be not significantly different from control (Fig. 6B).

**Inhibition of \( K^+ \) channels by nicotine is rapid.** If nicotine is acting as blocker, there is some expectation that the \( K^+ \) channel inhibition would be rapid (Cooper et al. 2003). Consistent with this, the onset of the nicotine-evoked response at \(-10 \text{ mV} \) appeared to be at least 1 s during certain pressure-application experiments (e.g., see Fig. 3C or Fig. 4, A and B). To more accurately test the kinetics of the nicotine-mediated block, we pressure-apply agonist during a step protocol designed to repeatedly activate \( I_{\text{K}} \). The initial experiment involved delivering a 20-s, 8-Hz train of 100-ms voltage steps from \(-50 \) to \(+30 \text{ mV} \) while pressure-applying either 10 mM nicotine or 100 \( \mu \text{M} \) epibatidine for 10 s, starting at the 4-s mark. Because of the pulse frequency during the stimulus train, currents were not leak subtracted (Fig. 7A, inset). Epibatidine, an alkaloid from the frog, *Epipodobates*, was selected as a control for any pressure artifacts both because of its structural similarity to nicotine (Badio and Daly 1994) and because epibatidine failed to activate a current in bag cell neurons at resting (\(-60 \text{ mV} \)) or depolarized potentials (\(-10 \text{ mV} \); \( n = 3 \); data not shown). Epibatidine was dissolved in nASW at as high a concentration as we found possible. \( I_{\text{K}} \) underwent use-dependent inactivation, judging by an immediate reduction in peak current, which fell quickly over the first 2 s but then slowed to a gradual and continuous decline for the remainder of the train (Fig. 7A). During pressure application of epibatidine (\( n = 7 \)), aside from the ongoing use-dependent inactivation, there was no apparent change in the magnitude of the peak current (Fig. 7A, filled circles, left inset). In contrast, when nicotine was introduced (\( n = 8 \)), the \( I_{\text{K}} \) dropped suddenly but then returned to preblock levels almost as quickly when the nicotine application had ceased (Fig. 7A, open circles, right inset). This reduction took place within 250 ms of pressure-application onset and reached a maximum by \(-3 \text{ s} \) of nicotine delivery (Fig. 7B, right); such changes were not evident during epibatidine application (Fig. 7B, left). The average maximal decrease in peak current due to nicotine was significantly larger, at \(-550 \) pA, compared with that in epibatidine, at \(-15 \) pA (Fig. 7C).

Subsequently, experiments were undertaken to more closely examine the possibility that nicotine was hastening the use-dependent inactivation of \( I_{\text{K}} \). Specifically, we added nicotine alone and then later compared the extent of block with and without continuous stimulation up to that point. All neurons were first given a single, 100-ms step from \(-50 \) to \(+30 \text{ mV} \) to elicit \( I_{\text{K}} \), and then 5 min later, 10 mM nicotine was pressure-applied for 10 s. For nonstimulated cells, a second, solitary step to \(+30 \text{ mV} \) was delivered 8 s after the onset of nicotine, whereas for stimulated cells, use-dependent inactivation was brought about by an 8-Hz, 5-s train of steps to \(+30 \text{ mV} \) that was started 3 s after nicotine was introduced. The final step of the train stimulus was delivered at the same time as when the single step was given to nonstimulated neurons (Fig. 7D, top). The step prior to nicotine was given to all neurons to provide both a means to normalize some of the ensuing data and to be more certain that any differences were not due to cell-to-cell variability in current density. The degree of \( K^+ \) current inhibition in nicotine was greater with ongoing use-dependent inactivation from the stimulation (Fig. 7D, bottom right) than without stimulation (Fig. 7D, bottom left). In particular, compared with the peak current from the step prior to nicotine, there was a significantly greater decrease by the final step of the train stimulus in nicotine (stimulated; \( n = 5 \)) than by the solitary step in nicotine (nonstimulated; \( n = 5 \); Fig. 7E, top). Moreover, the density of the current evoked by the final step of the train stimulus in nicotine was significantly smaller than that of the solitary step in nicotine (Fig. 7E, bottom).

![Fig. 6](https://fjphysiol.physiology.org/lookup/doi/10.1152/jn.00816.2015)

**Fig. 6.** Nicotine inhibits both components of the delayed-rectifier \( K^+ \) current. A: in a whole cell voltage-clamped cultured bag cell neuron, the biexponential tail current of \( I_{\text{K}} \) is apparent following a 100-ms step to \(+30 \text{ mV} \) from a holding potential of \(-50 \text{ mV} \) (bottom). A two-term exponential fit of the tail current yields fast (\( \tau_{\text{fast}} \)) and slow (\( \tau_{\text{slow}} \)) time constants. In control (top), the current reflects an average of 5 steps, with the approximate placement of \( \tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) as indicated. After 10 min of 10 mM nicotine (Nic), the magnitude of \( I_{\text{K}} \) was smaller (middle), although the tail current was similar to control. Scale bars apply to both traces. B: despite a reduced \( I_{\text{K}} \) magnitude with nicotine, the summary data reveal no significant difference between control and nicotine for either \( \tau_{\text{fast}} \) or \( \tau_{\text{slow}} \) (both unpaired Student’s \( t \)-test). Numbers in bars indicate number of neurons.
Finally, we also compared two groups of neurons where either 10 mM nicotine or simply nASW was pressure-applied for 10 s and the 8-Hz, 5-s train of steps to +30 mV was given 3 s into the pressure application. The peak current evoked by the first pulse was divided into itself as well as all of the subsequent currents elicited by the train stimulus for a given neuron. These data were then averaged and fit with a two-term exponential function. Whereas the slow time constant from the fits was similar between the two groups (data not shown), the fast time constant showed a difference. Specifically, the use-dependent inactivation in nicotine was significantly quicker than in nASW ($\tau_{\text{fast-nicotine}} = 98.42 \pm 10.98$ ms, $n = 5$, vs. $\tau_{\text{fast-nASW}} = 223.7 \pm 64.15$ ms, $n = 8$; $P < 0.02$, Mann-Whitney U-test).

Short-term nicotine pretreatment increases excitability. The effects of nicotine on steady-state current at depolarized potentials, as well as on voltage-gated $I_A$ and $I_K$, is likely to have physiological consequences. Therefore, we tested whether pretreating cultured bag cell neurons for 10 min with 10 mM nicotine altered action potential dynamics or changed the ability to fire action potentials to a prolonged stimulus. Membrane potential recordings were made from neurons, and bias current was used to set them at $-60$ mV (see MATERIALS AND METHODS, Sharp-electrode current-clamp recording). For the pretreated cells, nicotine caused a 15.1 ± 6.9-mV depolarization (n = 9; data not shown) that recovered completely to $-60$ mV by the end of the 10 min. Our prior work, involving pressure application or brief periods of perfusion, showed that nicotine resulted in a change in membrane potential from $-60$ mV that was transient due to desensitization (White et al. 2014). Thus, in the present study, by the end of the 10-min nicotine pretreatment, any ionotropic receptors were closed and desensitized, and the only effect remaining was that of nicotine blocking mainly voltage-gated $K^+$ currents.

A 1-nA, 50-ms stimulus (Fig. 8A, bottom) was delivered to elicit a single action potential from $-60$ mV. In control neurons (n = 9), the action potential took $\sim27$ ms to reach a peak amplitude of $\sim75$ mV with a half-width of $\sim8$ ms (Fig. 8A, middle). However, in parallel neurons treated with nicotine (n = 9), the action potential reached peak in a faster time of $\sim20$ ms, with a larger magnitude of $\sim90$ mV and a broader half-width of $\sim17$ ms (Fig. 8A, top). On average, all three action potential parameters were significantly different in nicotine (Fig. 8C–E). Also, the current required to evoke a single action potential was reduced by nicotine (0.78 ± 0.08 nA in control vs. 0.38 ± 0.04 nA in nicotine; $P < 0.01$, Mann-Whitney U-test) without significantly affecting the input resistance, as determined from a resting potential of $-60$ mV by a $\sim50$-pA current pulse (308 ± 46 MΩ in control vs. 378 ± 38 MΩ for nicotine; $P > 0.05$, Mann-Whitney U-test).

To investigate possible changes to excitability brought about by nicotine, neurons were subjected to a 10-s depolarizing current pulse of between 200 and 800 pA, which corresponded to the observed rheobase for each given cell (Fig. 8B, bottom). Under control conditions, the 10-s current generated an initial burst that averaged fewer than 10 action potentials, followed by silence for the remainder of the stimulus (Fig. 8B, middle, and E). Conversely, the excitability of neurons that saw nicotine was increased, based on continuous firing during the 10-s current (Fig. 8B, top). The output following nicotine treatment was a significantly larger action potential production, with a mean of over 50 spikes (Fig. 8E).

DISCUSSION

Nicotine is generally considered an agonist that opens ionotropic cholinergic receptors (Albuquerque et al. 2009; Gardner et al. 1984; Langley 1905). Using Aplysia bag cell neurons, we have now shown that nicotine can cause apparent inward current by reducing one or more $K^+$ currents. Consistent with $K^+$ channel closure, this response is accompanied by a decline in membrane conductance, is prominent at depolarized potentials, is prevented by intracellular perfusion with Cs$^+$-based intracellular saline, and results in the appearance of negative slope in the I/V relationship. Exposure of bag cell neurons to nicotine also leads to changes in excitability that are in keeping with a lessening of $I_A$ and $I_K$. A decrease in voltage-gated $K^+$ channels, by either regulation or direct block, can have profound effects on neuronal properties (Byrne and Kandel 1996; Levitan and Kaczmarek 2002); accordingly, we observed faster spike onset and larger/broader action potentials, as well as a greater number of spikes, in response to stimulation.

Although the evidence mainly points to nicotine acting as a $K^+$ channel blocker, a genuine nicotinic receptor could be involved. If such a receptor exists, it does not appear to use classical metabotropic signaling, given the inability of the G protein blocker GDPβS to alter the nicotine-evoked current at $-10$ mV. Furthermore, it is unlikely that the nicotine response described in this report is due to activation of either of the previously characterized bag cell neuron cholinergic receptors, i.e., the distinct inward currents induced by acetylcholine or nicotine at $-60$ mV (White and Magoski 2012; White et al. 2014). Both of those responses are associated with an increase in membrane conductance, are unaffected by intracellular Cs$^+$ perfusion, are inwardly rectifying with depolarization, and do not pass current at $-10$ mV. Whereas the present study finds that none of the established nicotinic receptor antagonists impact the nicotine-evoked response at $-10$ mV, our prior work showed that mecamylamine antagonizes both the acetylcholine- and nicotine-elicited currents at $-60$ mV, whereas α-conotoxin Imi and hexamethonium exclusively inhibit the acetylcholine response (White and Magoski 2012; White et al. 2014). Finally, the nicotine-evoked response at $-10$ mV has a Hill slope of $\sim1$ and an EC$_{50}$ of $\sim1.5$ mM; this is dissimilar from the current elicited by nicotine at $-60$ mV, which presents a Hill slope of $\sim2.5$ and an EC$_{50}$ of $\sim250$ μM (White et al. 2014).

Cockroach dorsal unpaired median neurons also respond to nicotine with a decrease-conductance current (Courjaret and Lapied 2001; Thany et al. 2008). Like that in Aplysia, this current is $K^+$ permeable and sensitive to intracellular Cs$^+$ perfusion; however, unlike the Aplysia current, the Periplanta current is inhibited by mecamylamine and α-conotoxin Imi. Those authors did not directly test nicotine on $K^+$ currents; rather, they concluded that nicotine binding closed a constitutively active atypical nicotinic receptor. In rat striatal neurons, nicotine and the nicotinic agonist dimethylphenyl pyperazinium inhibit $I_K$ but not $I_A$ (Hamon et al. 1997). Although this effect is not sensitive to mecamylamine, hexamethonium, δ-tubocurarine, or the G/G$_o$ protein inhibitor pertussis toxin, a nicotine receptor is still implicated based on a block by the
competitive antagonist dihydro-β-erythroidine. These findings are disparate from those in bag cell neurons, where nicotine reduces multiple K⁺ currents: steady state at −10 mV as well as Iₖ and Iₖr.

Although nicotinic receptors typically function solely as ion channels, there are a few cases of coupling to second messengers. For mouse myotubes, nicotine mediates an increase in intracellular Ca²⁺. For mouse myotubes, nicotine mediates an increase in current maintained a stable decay. Arrows indicating Fig. 7. Inhibition of K⁺ current by nicotine has a rapid time course. A: Iₖ was evoked from cultured bag cell neurons under whole cell voltage-clamp with 100-ms steps from −50 to +30 mV delivered at 8 Hz for 20 s. Insets: overlays of currents induced by the train when either 100 μM epibatidine (epi; left) or 10 mM nicotine (Nic; right) were introduced about one-third into the duration of the stimulation. Scale bars apply to both sets of traces. Graph shows peak current vs. time for the 2 cells; use-dependent inactivation was rather abrupt at the outset but slowed to a more gradual decline by −2 s. Approximately 4 s into the train, a 10-s pressure application of agonist began (at horizontal bar). In conjunction with nicotine (open circles), the peak outward current was reduced within −250 ms. To control for pressure artifacts, 100 μM of the structurally similar amine epibatidine (filled circles) was pressure ejected onto a second neuron, but the peak current had a rapid time course. B: overlays of currents induced by the train when either 100 μM epibatidine (epi; left) or 10 mM nicotine (Nic; right) were introduced about one-third into the duration of the stimulation. Scale bars apply to both sets of traces. Graph shows peak current vs. time for the 2 cells; use-dependent inactivation was rather abrupt at the outset but slowed to a more gradual decline by −2 s. Approximately 4 s into the train, a 10-s pressure application of agonist began (at horizontal bar). In conjunction with nicotine (open circles), the peak outward current was reduced within −250 ms. To control for pressure artifacts, 100 μM of the structurally similar amine epibatidine (filled circles) was pressure ejected onto a second neuron, but the peak current had a rapid time course. B: overlays of currents induced by the train when either 100 μM epibatidine (epi; left) or 10 mM nicotine (Nic; right) were introduced about one-third into the duration of the stimulation. Scale bars apply to both sets of traces. Graph shows peak current vs. time for the 2 cells; use-dependent inactivation was rather abrupt at the outset but slowed to a more gradual decline by −2 s. Approximately 4 s into the train, a 10-s pressure application of agonist began (at horizontal bar). In conjunction with nicotine (open circles), the peak outward current was reduced within −250 ms. To control for pressure artifacts, 100 μM of the structurally similar amine epibatidine (filled circles) was pressure ejected onto a second neuron, but the peak current had a rapid time course.

Although nicotinic receptors typically function solely as ion channels, there are a few cases of coupling to second messengers. For mouse myotubes, nicotine mediates an increase in intracellular Ca²⁺ that necessitates both Na⁺ influx through the nicotinic receptor and inositol triphosphate production but is not affected by nicotinic antagonists or pertussis toxin (Grassi et al. 1993). Activation of α7-nicotinic receptors in rat microglia also elevates Ca²⁺, but it is not associated with any form of ionic current (Suzuki et al. 2006). Similarly, in specific Aplysia neurons, binding of nicotine to Cl⁻ permeable nicotinic receptors turns on lipoxygenase and can be prevented by pertussis toxin but does not require Cl⁻ influx per se. (Tieman et al. 2001). Such examples aside, these transduction pathways probably do not play a role in the nicotine-mediated effects described in the present study. GDPβS does not modify the nicotine-evoked response at −10 mV, and the rapid nature by which nicotine inhibits Iₖ (within 250 ms) seems too brief for second messenger-based signaling. Our laboratory and others have found that currents or membrane potential changes brought about by metabotropic receptors in Aplysia neurons...
operate on a timescale of seconds to minutes, as opposed to milliseconds (Bolshakov et al. 1993; Fisher et al. 1993; Tam et al. 2011; White and Magoski 2012). Parenthetically, we reported earlier that nicotine fails to alter bag cell neuron intracellular Ca\(^{2+}\) under voltage clamp (White et al. 2014).

Rather than a nicotinic receptor, the pharmacological data largely indicate block of K\(^+\) channels as the mechanism underlying both the nicotine-evoked response at \(-10\) mV and the reduction of \(I_A\) and \(I_K\) by nicotine in bag cell neurons. In addition, both time constants that describe the \(I_K\) tail current are unaltered by nicotine, suggesting that the two delayed rectifiers comprising \(I_K\) were equally blocked. A-type and inward-rectifier K\(^+\) currents are inhibited by nicotine in canine ventricular myocytes or when the corresponding genes (\(K_v4.3, K_v\text{IR}2.1, K_v\text{IR}2.2\)) are expressed in oocytes (Wang et al. 2000a, 2000b). Although those studies do not denote a time course for the action of nicotine, they do report that nicotine enhances use-dependent inactivation and that the block is not prevented by mecamylamine. K\(^+\) channels in rat tail artery smooth muscle cells are also sensitive to nicotine, i.e., millimolar levels block delayed-rectifier currents in a near-instantaneous and fully reversible manner (Tang et al. 1999). Unsurprisingly, the effect of nicotine on smooth muscle is not altered by dihydro-\(\beta\)-erythroidine.

The inhibition of bag cell neuron \(I_A\) and \(I_K\) following bath application or perfusion of nicotine, with no intervening test pulses, suggests the drug may bind to K\(^+\) channels that are closed. That stated, we also observe a seeming acceleration of \(I_K\) use-dependent inactivation by nicotine. Specifically, giving nicotine prior to repetitive stimulation of \(I_K\) results in a greater rundown of the current compared with unstimulated neurons that are exposed to nicotine or cells that receive control applications of nACh. Similar tonic and use-dependent blocks are reported by Wang et al. (2000a) for ventricular myocytes. With respect to the nicotine-evoked response at \(-10\) mV, this represents the closure of steady-state K\(^+\) channels that are open when bag cell neurons are held at \(-10\) mV. At this voltage, \(I_A\) will fully inactivate in a short period of time, but a small proportion of \(I_K\) is still available (Strong 1984; Strong and Kaczmarek 1986). Hence, nicotine may affect some fraction of \(I_K\) that has not inactivated and/or a leak K\(^+\) channel. However, in keeping with a more specific action on voltage-dependent K\(^+\) currents, the negative-slope current appears only as the membrane potential approaches \(-30\) mV. Moreover, nicotine does not change input resistance measured from \(-60\) mV, where it is likely that \(I_A\) is completely closed and there is only modest activation of \(I_K\), leaving few channels for nicotine to block. Nicotine may be acting like TEA, a classical blocker (Hille 2001; Lorente de Nó 1949) which also inhibits voltage-gated K\(^+\) channels at millimolar concentrations in neurons from *Aplysia*, including bag cell neurons (Hermann and Gorman 1981; Quattrocchi et al. 1994; Strong and Kaczmarek 1986), as well the pulmonate molluscs, *Helix* and *Onchidium* (Hagiwara and Saito 1959; Neher and Lux 1972). Potentially, the N-methyl group of the pyrrole ring on nicotine may be occluding the outer pore region of the channels in a fashion similar to the N-ethyl groups of TEA (MacKinnon and Yellen 1990).

During the afterdischarge, a reduction of K\(^+\) currents by phosphorylation enhances bag cell neuron excitability (Kaczmarek et al. 1980; Kaczmarek and Strumwasser 1984; Strong 1984; Strong and Kaczmarek 1986; Zhang et al. 2004), and this is mimicked by exogenous nicotine. The negative-slope \(I/V\) that results from nicotine block of K\(^+\) channels markedly elevates the spiking response to prolonged current injection (Beilby 1986; Lüthi et al. 1997; Shen and Johnson 2013; Wilson and Wachtel 1974). Also, the speeding up of action potential generation in nicotine is ostensibly due to the reduction in \(I_A\), which normally serves to limit action potential initiation by activating near resting potential (Connor and Stevens 1971; Gustafsson et al. 1982; Strong 1984). Finally, the drop in \(I_K\) induced by nicotine likely enhances spike height and width, consistent with a role for delayed-rectifier K\(^+\) currents in controlling action potential dynamics at more depolarized voltages (Connor and Stevens 1971; Hagiwara and Saito 1959; Klein and Kandel 1978; Quattrocchi et al. 1994; Strong and Kaczmarek 1986).

The consequences of nicotine exposure in bag cell neurons may be applicable to the noncanonical effects of nicotine in both the heart and brain of other organisms, particularly in cases of nicotine poisoning, which often involve higher doses compared with that from smoking (Mayer 2014). For *Aplysia*, the shoreline marine environment has the potential to be contaminated with nicotine/neonicotinoids from pesticide runoff (Dondero et al. 2010; Felsot et al. 2002). In turn, this could alter reproductive outcomes in the mollusc by chronically blocking bag cell neuron K\(^+\) channels.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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

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