Persistent Ca\textsuperscript{2+} Current Contributes to a Prolonged Depolarization in Aplysia Bag Cell Neurons

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INTRODUCTION


The bag cell neurons of the marine mollusk, Aplysia californica, initiate reproduction via a striking activity-dependent change in excitability known as the afterdischarge (Kupfermann 1967; Kupfermann and Kandel 1970; Pinsker and Dudek 1977). Following transitory synaptic input, these neuroendocrine cells fire action potentials for around 30 min and secrete egg-laying hormone into the circulation (Chiu et al. 1979; Russo and Hounsgaard 1996; Zhang and Harris-Warrick 2005). However, as originally found in Helix neurons by Eckert and Lux (1976), pacemaker current can also be attributed to the voltage-dependent activation of persistent Ca\textsuperscript{2+} current and is subject to modulation. Such synergy between currents may represent a common means of achieving activity-dependent changes to excitability.

METHODS

Animals and cell culture

Adult Aplysia californica weighing 150–500 g were obtained from Marinus (Long Beach, CA) or Santa Barbara Marine Biologicals...
Whole cell, voltage-clamp recordings

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 ID borosilicate glass capillaries (TW150F-4, World Precision Instruments; Sarasota, FL) and had a resistance of 1–2 MΩ when filled with either intracellular saline. Pipette junction potentials were nulled, and subsequent to seal formation, pipette capacitive currents were cancelled. Following break-through, neuronal capacitance was also cancelled, and the series resistance (3–5 MΩ) compensated to 80% and monitored throughout the experiment. Current was filtered at 1 kHz with the EPC-8 built-in Bessel filter and sampled at 2 kHz using a Digidata 1322A A/D converter (Axon Instruments/Molecular Devices; Sunnyvale, CA), a computer, and Clampex software (version 8.2, Axon Instruments). Voltage stimuli were delivered with either Clampex or a S88 stimulator (Grass; Warwick, MA).

Ca2+ currents were isolated using Ca2+-Cs+-TEA ASW, where the Na+ was replaced with TEA and the K+ with Cs+ (composition in mM: 460 TEA-Cl, 10.4 CsCl, 55 MgCl2, 11 CaCl2, and 15 HEPES, pH 7.8 with CsOH). The procedure also employed a Cs-based intracellular saline with a composition (in mM) of 70 CsCl, 10 HEPES, 11 glucose, 10 glutathione, 5 ethyleneglycol bis (aminoethyl ether) tetraacetic acid (EGTA), 500 aspartic acid, 5 ATP (grade 2, disodium salt; A3377, Sigma), and 0.1 GTP (type 3, disodium salt; A9550, Sigma). Voltage stimuli were delivered with either Clampex or a S88 stimulator (Grass; Warwick, MA).

Ca2+ imaging

The Ca2+-sensitive dye, fura-PE3 (K+ salt, 0110; Teflabs, Austin, TX) (Vorn德拉n et al. 1995), was injected using a PMI-100 pressure microinjector (Dagan, Minneapolis, MN), while simultaneously monitoring membrane potential with the Axoclamp. Microelectrodes (as per sharp-electrode current clamp) had a resistance of 15–30 MΩ when tip-filled with 10 mM fura-PE3 and backfilled with 3 M KCl. Filling neurons with an optimal amount of dye, estimated at 50–100 μM, required 3–10 300- to 900-ms pulses at 50–100 kPa. After injection, neurons were allowed to equilibrate for ≥30 min. Imaging was performed in nASW using a Nikon TS100-F inverted microscope (Nikon, Mississauga, ON, Canada) equipped with Nikon Plan Fluor objective (BP231-1, Fisher) and the tight-seal, current-clamp bridge (Nikon, Mississauga, ON, Canada) coupled to the microscope with a UV-grade liquid-light guide. Excitation wavelengths of 340 and 380 nm were controlled by a Chroma Technologies LP-1000 intensified charge coupled device camera. The camera black level was set prior to an experiment such that at a gain of 1, and with no light going to the camera, there was a 50:50 distribution of blue and black pixels on the image display. The camera intensifier voltage was set based on the initial fluorescence intensity of a neuron at the start of the experiment and maintained constant thereafter. Fluorescence intensities were sampled every 10 s from regions of interests (ROIs) defined over the soma and averaged four to eight frames per acquisition. The emission following 340 and 380 nm excitation was ratioed (340/380) to reflect free intracellular Ca2+ and saved for subsequent analysis. Black level adjustment, image acquisition, frame averaging, emitted light ROI sampling, and ratio calculations were performed by ImageMaster Pro.

Reagents and drug application

In cases where Ni2+ was used to subtract leak current, it was applied via a gravity-driven perfusion system before the stimulus was given. In experiments where Ni2+ was applied after a prolonged depolarization was elicited under sharp-electrode current clamp, it was perfused following the stimulus. Dimethyl sulfoxide (DMSO; BP231-1, Fisher) was used as the vehicle for phorbol 12-myristate 13-acetate (PMA; P8139, Sigma). Bag cell neurons were pretreated for 20–30 min in 0.5% DMSO or 100 nM PMA.

Analysis

The current-voltage relationship of rapid Ca2+ or Ba2+ current was ascertained using Clampfit software (version 8.2, Axon Instru-
ments) by measuring peak current between cursors set as close as possible to the start and end of leak-subtracted traces. Current was normalized to cell size by dividing by neuronal capacitance (obtained from the EPC-8 whole cell capacitance compensation circuitry) and plotted against voltage. Activation curves were determined by dividing the current elicited at each voltage step by the maximum current elicited during the protocol (+10 mV; see results for details). This was averaged across cells at a given step voltage, plotted against that voltage, and fit with a Boltzmann equation in Origin (version 7, OriginLab; Northampton, MA). Pseudo-steady-state inactivation curves were determined by first delivering a 10-s inactivating step, then testing the current by stepping to +10 mV and subsequently dividing all test currents by the maximal test current (evoked from an inactivation step of −90 or −60; see results for details). This was averaged across cells at a given inactivating voltage, plotted against that voltage, and again fit with a Boltzmann. The fits provided the half-maximal voltage (V_{1/2}) of activation (the voltage required to recruit half of the maximum current) or inactivation (the voltage at which half of the current remains for recruitment) and the slope factor (k; the amount of voltage required to change the current e-fold).

The current-voltage relationship of persistent Ca^{2+} or Ba^{2+} currents was also established using Clampfit. Following leak subtraction by Ni^{2+} block, cursors were placed at the beginning and end of the period in the current trace prior to stimulus application, and the mean between these two cursors was taken as the holding current. Cursors were also placed near the end of the voltage step, separated by either 1 s for the 10- and 30-s steps or 10 s for the 1-min steps. The mean between these two cursors was taken as the current elicited by the step. The difference between the mean current during the step and the mean holding current was taken as the persistent current. Current was normalized to cell size by dividing by the whole cell capacitance and plotted against step voltage.

Clampfit was used to determine peak amplitude of either an evoked depolarization or I_{PD}. Cursors were placed at baseline voltage or current, prior to delivery of the stimulus, as well as at peak voltage or current amplitude during or after the stimulus. The difference between the two cursor values was taken as the amplitude. The prolonged depolarization current was normalized to cell capacitance. For display, voltage traces and I_{PD} were filtered off-line to 10–20 Hz using Clampfit. The very slow nature of the membrane potential responses was also established using Clampfit. Following leak subtraction in Origin (version 7, OriginLab; Northampton, MA), pseudo-voltage, plotted against that voltage, and fit with a Boltzmann equation, providing the half-maximal voltage (V_{1/2}) of activation (the voltage required to change the current e-fold). This was averaged across cells at a given inactivating voltage, plotted against that voltage, and again fit with a Boltzmann. The fits provided the half-maximal voltage (V_{1/2}) of activation (the voltage required to recruit half of the maximum current) or inactivation (the voltage at which half of the current remains for recruitment) and the slope factor (k; the amount of voltage required to change the current e-fold).

For intracellular Ca^{2+}, Origin was used to import and plot ImageMaster Pro files as line graphs. The steady-state value of the baseline 340/380 ratio under simultaneous voltage clamp at −60 mV was compared with the ratio from regions that had reached a peak or new steady state during a 1-min step to a depolarized potential. Averages of both regions were determined by eye or with adjacent-averaging. Change was expressed as a percent change (% change 340/380) of the new ratio over the baseline ratio.

Data are presented as the means ± SE as calculated using either Origin or Instat (version 3.05; GraphPad Software; San Diego, CA). Statistical analysis was performed using Instat. The Kolmogorov-Smirnov method was used to test data sets for normality. A one-sample t-test was used to determine if the mean of a single group was different from a mean of zero. Student’s paired or unpaired t-test (with the Welch correction as required) or the Mann-Whitney test was used to compare differences between two means, while either a standard one-way ANOVA and the Student-Newman-Keuls multiple-comparisons post hoc test or a Kruskal-Wallis nonparametric ANOVA and Dunn’s multiple comparisons post hoc test were used to compare differences between multiple means. Means were considered significantly different if the P value was <0.05.

RESULTS

Rapid voltage-gated Ca^{2+} current in cultured bag cell neurons

Bag cell neuron Ca^{2+} current has been examined previously (Conn et al. 1989; DeRiemer et al. 1985b; Hung and Magoski 2007; Zhang et al. 2008); however, the information on permeation and fundamental biophysics is less than complete (Fieber 1995). Employing a Ca^{2+}-aspartate-based intracellular saline and Ca^{2+}-Cs^{+}-TEA ASW in the bath, cultured bag cell neurons were whole cell voltage-clamped at −60 mV and Ca^{2+} currents evoked with 200-ms square pulses from −60 to +40 mV in 10-mV increments. What we designate here as the rapid Ca^{2+} current was fast activating, strongly voltage-dependent, moderately inactivating during the pulse, and maximal at +10 mV (Fig. 1A, left). As our laboratory has demonstrated previously (Hung and Magoski 2007), these currents were abolished by 10 mM of the general Ca^{2+} channel blocker, Ni^{2+} (Byerly et al. 1985; McFarlane and Gilly 1998) (Fig. 1A, right). Compared with control (n = 9), there was an absence of inward current at all voltages following Ni^{2+} block (n = 6) (Fig. 1B). In certain subsequent experiments, we used 10 mM Ni^{2+} to block Ca^{2+} currents under voltage or current clamp.

Ca^{2+} channels are permeable to other divalent cations, such as Ba^{2+} (Hagiwara et al. 1974; Hille 2001). Substituting Ba^{2+} for Ca^{2+} typically results in larger currents and in some instances a negative shift in the V_{1/2} voltage of activation (Byerly et al. 1985; Hess et al. 1986). Ba^{2+} currents were recorded as per Ca^{2+} currents, but with Ba^{2+}-Cs^{+}-TEA ASW externally. Particularly at test potentials more negative than 0 mV, the Ba^{2+} current was larger than the Ca^{2+} current (n = 7; Fig. 1C). Contributing to this apparent increase in amplitude was a negative shift in the peak Ba^{2+} current to 0 mV (Fig. 1D). The latter result leads us to consider the possibility that replacing Ca^{2+} with Ba^{2+} as a charge carrier may have altered the voltage dependence of activation and/or inactivation, an observation we have made previously when studying cation channels (Geiger et al. 2009).

For activation, both Ca^{2+} and Ba^{2+} currents were normalized to the current evoked during the pulse to +10 mV and plotted against test pulse voltage. A Boltzmann fit of these relationships showed a left-shifted activation with Ba^{2+} as indicated by the more negative V_{1/2} of activation (about −6 mV in Ca^{2+} vs. about −19 mV in Ba^{2+}; n = 8 and 7; Fig. 2A). The k values reflected a small increase in sensitivity with Ba^{2+} (near 5 in Ca^{2+} vs. near 4 in Ba^{2+}). For steady-state inactivation, neurons were held at either −60 mV (for Ca^{2+}) or −90 mV (for Ba^{2+}), and prior to a +10-mV test pulse, currents were inactivated with 10-s steps to +10 mV in 10-mV increments. Fitting a Boltzmann function to the inactivation curves revealed that Ba^{2+} currents (n = 8) inactivated at a much hyperpolarized voltage compared with Ca^{2+} currents (n = 5; Fig. 2B). This was reflected by the more negative V_{1/2} of inactivation (around −14 in Ca^{2+} vs. around −30 in Ba^{2+}) and was accompanied by a slight increase in sensitivity with a lowering of the k value (−10 in Ca^{2+} vs. −8 in Ba^{2+}). For simultaneous comparison of activation and inactivation, the curves derived from the Boltzmann fits of the Ca^{2+} current are re-plotted in Fig. 2C. A reasonable degree of overlap was evident between the two curves with a point of intersection at close to −9 mV.
The prolonged depolarization in bag cell neurons is driven by a voltage-independent, nonselective cation current that lasts for 3–5 min under voltage clamp (Hung and Magoski 2007). Because the prolonged depolarization lasts upward of 30 min, we hypothesized that the response is maintained by a persistent voltage-dependent Ca\(^{2+}\) current. To test this, cultured bag cell neurons were voltage-clamped at −60 mV and stimulated in 10-mV increments with 10- or 30-s square pulses to −20 mV \((n=7; \text{Fig. } 3, \text{A} \text{ and } \text{B})\) or 1-min pulses to −30 mV \((n=6; \text{3C})\). Currents were leak subtracted by delivering the voltage steps before and after 10 mM Ni\(^{2+}\) block, then subtracting the current in Ni\(^{2+}\) from that under control. During all steps, Ni\(^{2+}\) eliminated much of the current, but subtraction revealed a small, inward, voltage-dependent current after any fast, partial inactivation at the onset of the step. Particularly over the 1-min pulse, the current was essentially stable throughout. Mean currents taken near the end of the step (see METHODS for details) were not significantly different between the three durations at a given voltage except for the step to −50 mV for 10 s versus 30 s or 1 min (Fig. 3D).

As an independent assessment of Ca\(^{2+}\) entry under more physiological conditions, fura-loaded cultured bag cell neurons were ratiometrically imaged in nASW while simultaneously voltage-clamped at −60 mV. Delivery of 1-min step depolarizations from −50 to −20 mV in 10-mV increments induced clear elevations of intracellular Ca\(^{2+}\) as indicated by an increase in the 340/380 fura-PE3 ratio \((n=17; \text{Fig. } 4\text{A})\). Changes in Ca\(^{2+}\) were most prominent, although more transitory, during pulses to −30 and −20 mV; however, depolarization to −40 and even −50 mV elicited resolvable Ca\(^{2+}\) increases. The mean change in Ca\(^{2+}\) evoked by stepping to −20 mV was significantly different from that produced by pulses to −50 or −40 mV; this was also the case for the step to −30 mV compared with −50 mV (Fig. 4B). Overall there was a clear nonlinear trend for greater intracellular Ca\(^{2+}\) with greater depolarization, indicative of voltage-gating mediating the response. These data suggest that in a normal ionic environment, even relatively small alterations to membrane potential can permit Ca\(^{2+}\) entry.

Given that the rapid current was relatively larger over the negative range of test voltages with Ba\(^{2+}\) substituted for Ca\(^{2+}\) (see Fig. 1D), we examined the persistent current using Ba\(^{2+}\) as a charge carrier. Because there was relatively little difference between Ca\(^{2+}\) currents at the three extended test pulse durations (see Fig. 3D), we used the 10-s step duration in this and subsequent experiments. Cultured bag cell neurons were stimulated from a holding potential of −60 to −20 mV in 10-mV increments under voltage clamp (Fig. 5A). Once normalized to cell capacitance, the mean Ba\(^{2+}\) current \((n=7)\) was larger when compared with Ca\(^{2+}\) current \((n=6)\) recorded in separate experiments, with the difference reaching significance at −30 and −20 mV (Fig. 5B). This indicates the persistent current, like the rapid current, resembles a typical Ca\(^{2+}\) channel and shows a greater conductance with Ba\(^{2+}\) as the permeating ion.

Ni\(^{2+}\) does not inhibit the prolonged depolarization current but attenuates the prolonged depolarization itself

Hung and Magoski (2007) demonstrated that the initial current driving the prolonged depolarization \(.\text{IP}_{pd}\) was a voltage-independent nonselective cation channel. It is unlikely that the persistent Ca\(^{2+}\) current contributes directly to \(.\text{IP}_{pd}\); nevertheless, we tested this possibility by attempting to block \(.\text{IP}_{pd}\) with Ni\(^{2+}\). As per Hung and Magoski (2007), \(.\text{IP}_{pd}\) was evoked in cultured bag cell neurons voltage-clamped at −60 mV in nASW with a K\(^{+}\)-aspartate-based intracellular solution using a
5-Hz, 10-s train of 100-ms pulses to +10 mV (n = 5; Fig. 6A, left). This stimulus is analogous in frequency and duration to the train of synaptic input delivered to the intact cluster when triggering an afterdischarge (Kaczmarek et al. 1982; Magoski and Kaczmarek 2005). The posttrain current was similar to what we have reported previously, i.e., relatively slow onset with a near complete decay over the course of 10 min. This current was compared with \( I_{\text{PD}} \) elicited from different cells but with 10 mM Ni\(^{2+}\) perfused onto the neuron just after the train (n = 5; Fig. 6A, right). The data showed that Ni\(^{2+}\) did not block \( I_{\text{PD}} \), leaving the peak amplitude unchanged (Fig. 6B).

We used the fact that Ni\(^{2+}\) does not block \( I_{\text{PD}} \) to evaluate whether the persistent Ca\(^{2+}\) current plays a role in the maintenance of the prolonged depolarization after \( I_{\text{PD}} \) has diminished. Again as per Hung and Magoski (2007), prolonged depolarizations were evoked from cultured bag cell neurons under current clamp in nASW with a K\(^{+}\)-acetate-filled sharp electrode using a 5-Hz, 10-s train of action potentials. Once a depolarization had plateaued, 10 mM Ni\(^{2+}\) was added to the bath (n = 8; Fig. 7A). Perfusion of Ni\(^{2+}\) lead to a relatively rapid reduction of the prolonged depolarization magnitude with a near 75% percent recovery back to baseline (−60 mV) that reached the level of significance compared with a mean of zero (Fig. 7B).

Our study employed Ni\(^{2+}\) as a general Ca\(^{2+}\) channel blocker; however, Ni\(^{2+}\) also specifically blocks low-voltage-activated/T-type Ca\(^{2+}\) currents when used at comparatively low concentrations, including in the related mollusk, \textit{Lymnaea} (Fox et al. 1987; Lee et al. 1999; Yeoman et al. 1999). Based on biophysics, it is unlikely that a T-type Ca\(^{2+}\) current is involved in the generation or maintenance of the prolonged depolarization. In particular, T-type channels inactivate at voltages equivalent or more positive than resting potential, and when activated, they turn off in <200 ms (Carbone and Lux 1984; Fox et al. 1987). Nevertheless, to ensure that T-type Ca\(^{2+}\) current was not present, cultured bag cell neurons were again voltage-clamped using Cs\(^{+}\)-aspartate-based intracellular saline and Ca\(^{2+}\)-Cs\(^{+}\)-TEA ASW in the bath. Ca\(^{2+}\) currents were evoked with 200-ms square pulses from −60 to +40 mV in 10-mV increments from either a control holding potential of −60 mV (n = 10) or, to remove inactivation of any potential T-type current, −90 mV (n = 8). Consistent with an absence of T-type Ca\(^{2+}\) channels, the current-voltage relationships obtained from the two holding potentials were virtually identical (Fig. 7C). If a low-voltage-activated current was expressed, there would have been a distinct plateau in the relationship somewhere between −60 and −20 mV (Carbone and Lux 1984).

The block of an ongoing prolonged depolarization by Ni\(^{2+}\) suggests the persistent Ca\(^{2+}\) current plays a role in maintaining the response, but it does not provide information as to which current is at work during the initial phase. In an attempt to address this, prolonged depolarizations were elicited in control neurons (n = 8) versus cells that

**FIG. 2.** Activation and inactivation characteristics of rapid Ca\(^{2+}\) and Ba\(^{2+}\) currents. A: activation curves for Ca\(^{2+}\) and Ba\(^{2+}\) current. Currents are normalized to maximum current, plotted against voltage, and fit with a Boltzmann function. The activation curve with Ba\(^{2+}\) is shifted left as indicated by the lower \( V_{1/2} \), but there is little change in sensitivity, as reflected by similar \( k \) values. B: steady-state inactivation curves for Ca\(^{2+}\) and Ba\(^{2+}\) current. From a holding potential of either −60 mV (for Ca\(^{2+}\)) or −90 mV (for Ba\(^{2+}\)), the current is inactivated with a 10-s step to +10 mV in 10-mV increments followed by a 200-ms test pulse to +10 mV. The steady-state current evoked during each test pulse is divided by the maximal test pulse current (elicited from −60 or −90 mV for Ca\(^{2+}\) and Ba\(^{2+}\)), plotted against the corresponding inactivation step voltage, and fit with a Boltzmann function. Ba\(^{2+}\) inactivates at a more hyperpolarized voltage compared with Ca\(^{2+}\), indicated by its lower \( V_{1/2} \) and is somewhat more sensitive, as reflected by the lower \( k \) value. C: a re-plot of the Ca\(^{2+}\) current activation and inactivation curves, provided by the Boltzmann function, on the same graph and at the same scale. The same curves are removed for clarity. The activation curve shows a threshold of around −42 mV and intersects with the inactivation curve near −9 mV. Overlap between the 2 curves was evident, particularly between −40 and 0 mV.
were exposed to 10 mM Ni^{2+} immediately after the 5-Hz, 10-s train of action potentials (n = 8) (Fig. 7D). In both cases, the depolarization was evident and the difference between the two conditions did not reach significance (control: 9.8 ± 4.4 mV vs. Ni^{2+}: 10.5 ± 3.0 mV; P > 0.05, 2-tailed unpaired Student’s t-test). However, delivery of Ni^{2+} prior to development of the prolonged depolarization shortened the duration of the response. Compared with control, Ni^{2+}-exposed neurons largely recovered to the pre-stimulus membrane potential within 10 min (control: 16.3 ± 12.5% recovery vs. Ni^{2+}; 96.4 ± 3.6% recovery; P < 0.01, Mann-Whitney test). This is consistent with the cation channel being capable of depolarizing the neurons at the start of the response but nevertheless requiring subsequently recruitment of the Ca^{2+} current to keep the voltage depolarized.

PKC activation enhances both rapid and persistent Ca^{2+} current

It is established that activation of PKC augments bag cell neuron Ca^{2+} current (Conn et al. 1989; DeRiemer et al. 1985b; Strong et al. 1987; Zhang et al. 2008). Moreover, PKC activity is elevated shortly after the onset of the afterdischarge in intact bag cell neuron clusters (Wayne et al. 1999). We confirmed the effect of PKC on rapid Ca^{2+} currents evoked in cultured bag cell neurons with 200-ms square pulses from −60 to +40 mV in 10-mV increments following 30-min pretreatment in the vehicle, 0.1% DMSO (n = 9) or 100 nM of the PKC-activating phorbol ester, PMA (n = 8) (Castagna et al. 1982; DeRiemer et al. 1985a; Manseau et al. 2001). As expected, upregulation of PKC caused a marked enhancement of Ca^{2+} current compared with control (Fig. 8A, left and middle). The difference in

FIG. 3. Persistent voltage-activated Ca^{2+} current in cultured bag cell neurons. A and B: traces of Ca^{2+} currents evoked by 10- or 30-s square pulses from a holding potential of −60 to −20 mV in 10-mV increments. Currents are resolved by delivering the voltage steps before and after 10 mM Ni^{2+} block, then subtracting the current in Ni^{2+} from that under control. C: traces of Ca^{2+} currents evoked by 1-min square pulses from a holding potential of −60 to −30 mV in 10-mV increments. Again, Ni^{2+} subtraction is used to remove the leak. D: summary graph of mean persistent Ca^{2+} current normalized to cell capacitance. The persistent current is measured as the mean of the last 1 s during the 10- and 30-s steps or the last 10 s of the 1-min step. Mean currents are not significantly different, except at −50 mV between the 10-s and 1-min step, as well as between the 30-s and 1-min step (*P < 0.05, 1-way ANOVA, Student-Newman-Keuls multiple comparison post hoc test).

FIG. 4. Modest depolarizations elevate intracellular Ca^{2+} in cultured bag cell neurons. A: sample of intracellular Ca^{2+} as measured by 340/380 nm imaging of a fura PE3-loaded neuron bathed in nASW and simultaneously voltage-clamped at −60 mV. Ca^{2+} influx is evoked by 1-min square pulses to −50 mV through −20 mV in 10-mV increments as indicated. While the response is more long-lasting at −50 or −40 mV, stepping to −30 or −20 mV reliably produces a prominent peak of Ca^{2+} influx that subsequently decays. B: grouped imaging data of peak percentage change shows that intracellular Ca^{2+} increases in nonlinear manner as the membrane potential is made more positive, consistent with an underlying voltage-dependent process. Compared with the mean change during the −50-mV step, the responses reach the level of significance when the membrane potential is changed to −30 or −20 mV; in addition, the mean change at −40 mV is significantly different from that at −20 mV (Kruskal-Wallis nonparametric ANOVA, Dunn’s multiple comparison post hoc test).
average peak current, normalized to cell capacitance, between the two conditions was significant at all voltages except –60, –50, and +40 mV (Fig. 8A, right).

We next examined if the persistent current in those same PMA-responsive neurons also shared a sensitivity to PKC-dependent modulation. To test this, persistent Ca^{2+} currents were evoked by 10-s square pulses from –60 to –20 mV in 10-mV increments. The results showed that the presence of PMA lead to a larger persistent current (Fig. 8B, left and middle). The difference in the mean end-pulse current, normalized for cell size, reached the level of significance at –30 and –20 mV (Fig. 8B, right).

**PKC activation does not alter the prolonged depolarization elicited by an action potential train**

Knowing that the persistent Ca^{2+} current plays a role in the maintenance of the prolonged depolarization and this current is PKC sensitive, it follows that the prolonged depolarization should be PKC sensitive. This was examined by evoking the prolonged depolarization in current clamp with a 5-Hz, 10-s train of action potentials following 20-min pretreatment with 0.1% DMSO (n = 9) or 100 nM PMA (n = 9). Interestingly, the prolonged depolarization elicited under either condition was essentially the same (–10–12 mV; Fig. 9A). The difference in the average depolarization between the two groups was not significant (Fig. 9B). Our laboratory previously found that induction of either I_{PD} or the prolonged depolarization was actually limited or decreased when Ca^{2+} influx during the train was too great (Hung and Magoski 2007). Thus it is not surprising that following the PKC-induced enhancement of the rapid Ca^{2+} current, the prolonged depolarization remained unchanged even though the persistent current would also be augmented. Moreover, there is the issue of how PKC activation is timed. In the intact cluster, PKC would be upregulated subsequent to the delivery of the stimulus (Wayne et al. 1999), whereas our experimental conditions necessitate that PKC be turned on by PMA prior to the train.

**PKC activation enhances the prolonged depolarization elicited by a current ramp mimicking I_{PD}**

To avoid the confounding issue of PKC over-enhancing Ca^{2+} influx during the initiation of the prolonged depolarization, as well as any unknown effects on I_{PD}, the response was instead elicited in a manner that did not involve a train of action potentials. Specifically, a current ramp approximating an average I_{PD}, i.e., an inverted version of the inward current observed under voltage clamp, was delivered to cultured bag cell neurons under current clamp. Neurons were injected with the current ramp following 20-min pretreatment with 0.1% DMSO (n = 6) or 100 nM PMA (n = 5). The ramp depolarized the membrane potential with a time course that essentially corresponded to the duration of the current injection. Subsequent to the current, the membrane potential did not recover to baseline but rather underwent a phase of prolonged depolarization (Fig. 10A). The extent of the peak depolarization evoked during the current injection itself was not different in DMSO versus PMA. (Fig. 10B). However, the prolonged depolarization elicited after the current was significantly larger following activation of PKC by PMA (Fig. 10A, right, and C) compared with DMSO (Fig. 10A, left, and C).

**Discussion**

Ca^{2+} channels hold a privileged position of triggering plasticity and secretion (Mermelstein et al. 2001; Neher and...
Sakaba 2008) as well as passing inward current to affect excitability and activity (Eckert and Lux 1976; Metz et al. 2005; Wang et al. 2001). The bag cell neuron action potential is Ca$^{2+}$ dependent with the upstroke mediated by a rapid Ca$^{2+}$ channel designated as Apl-CaV1 based on partial cloning of the $\alpha$-subunit (Acosta-Urquidi and Dudek 1981; Kaczmarek and Stumwasser 1984; White and Kaczmarek 1997). PKC activity is upregulated 5 min into the afterdischarge (Wayne et al. 1999), which brings about the membrane insertion of a second species of rapid Ca$^{2+}$ channel, known as Apl-CaV2, to increase macroscopic Ca$^{2+}$ current (DeRiemer et al. 1985b; Zhang et al. 2008). These two currents have a similar voltage dependence, are weakly sensitive to nifedipine, and are blocked by millimolar levels of Ni$^{2+}$, Co$^{2+}$, or La$^{3+}$ (Hung and Magoski 2007; Strong et al. 1987). We now show that the bag cell neurons also express a persistent Ca$^{2+}$ current.

Both the rapid and persistent Ca$^{2+}$ current are enhanced when Ba$^{2+}$ replaces Ca$^{2+}$ as the charge carrier. This effect is widely reported for other Ca$^{2+}$-permeable channels (Friel and Tsien 1989; Geiger et al. 2009; Hagiwara et al. 1974; Hess et al. 1986; Tillotson 1979; Yue and Marban 1990). Ba$^{2+}$ is believed to have a lower binding affinity than Ca$^{2+}$ for the pore, resulting in greater mobility and conductance. In part, the larger rapid and persistent Ba$^{2+}$ currents observed in the present study may arise from a prominent left-shift in activation. Byerly et al. (1985) reported a near −15 mV shift in the $V_{1/2}$ of activation.

**FIG. 7.** Ni$^{2+}$ inhibits an ongoing prolonged depolarization. A: sample trace of a prolonged depolarization, recorded in nASW with a K$^{+}$-acetate filled sharp electrode, evoked (at bar) with a 5-Hz, 10-s train of action potentials. Addition of 10 mM Ni$^{2+}$ after the depolarization had plateaued leads to a reduction of the response. B: summary graph of percentage recovery back to baseline (−60 mV) after perfusion of Ni$^{2+}$. The recovery is significant compared with a mean of 0 (P < 0.01, 2-tailed 1-sample t-test). C: summary of peak Ca$^{2+}$ current normalized to capacitance evoked by 200-ms square pulses from −60 mV (●) or −90 mV (○). The 2 current-voltage relationships are very similar; in particular, there is a no plateau of over the negative test potentials when the neurons are held at −90 mV. A T-type Ca$^{2+}$ current would manifest as additional inward current activating at some point between −60 and −20 mV. D: top: control prolonged depolarization evoked by a train under sharp-electrode current-clamp shows little recovery over the duration of the recording period. Representative of n = 8. Bottom: when 10 mM Ni$^{2+}$ is applied (at bar) just after the stimulus has ended, the response still occurs but does not remain in the depolarized state. Instead, the membrane potential recovers over the course of several minutes. Representative of n = 8.
when using Ba$^{2+}$ to record rapid Ca$^{2+}$ currents from *Aplysia* neurons. They suggested that Ba$^{2+}$ impacts the voltage sensor by changing the external surface potential imposed on the channel. Regarding bag cell neurons, currents recorded by Fieber (1995), using Ba$^{2+}$ as a charge carrier and a CsCl-based internal, show an inactivation $V_{1/2}$ of $-30$ mV; however, that study did not address activation nor did it assess the current with Ca$^{2+}$ as charge carrier.

We confirmed that activation of bag cell neuron PKC increases the rapid Ca$^{2+}$ current (DeRiemer et al. 1985b; Zhang et al. 2008). At the concentration used in the present study, PMA is a specific and potent activator of bag cell neuron PKC (DeRiemer et al. 1985a; Manseau et al. 2001). The enhanced Ca$^{2+}$ current results in greater collective Ca$^{2+}$ influx, Ca$^{2+}$-induced Ca$^{2+}$ release, and peptide secretion during the after-discharge (Conn et al. 1989; Geiger and Magoski 2008; Loechner et al. 1992; Strong et al. 1987; Wayne et al. 1998). PKC-dependent modulation of rapid Ca$^{2+}$ current is well established in both invertebrate and vertebrate neurons (e.g., Dreijer and Kits 1995; Hammond et al. 1987; Yang and Tsien 1993). Indeed, the original report by DeRiemer et al. (1985b) on bag cell neurons was the first to demonstrate that PKC could alter Ca$^{2+}$ channel function. We now show that PKC has a similar effect on persistent Ca$^{2+}$ current with a near doubling of the density. There is little known regarding specific intracellular signaling molecules modulating persistent Ca$^{2+}$ current, although activation of adrenergic and serotonin receptors regulates these channels in hippocampal, spinal, and stomatogastric neurons (Cloues et al. 1997; Li et al. 2007; Perrier and Hounsgaard 2003; Zhang et al. 1995).

Application of Ni$^{2+}$ completely abolishes both the rapid and persistent Ca$^{2+}$ current in bag cell neurons. While millimolar concentrations of Ni$^{2+}$ will block most Ca$^{2+}$ channels (Byerly et al. 1985; McFarlane and Gilly 1998), that the two currents share Ni$^{2+}$ sensitivity suggests at the very least a common origin. Unfortunately, there are no adequate pharmacological tools to differentiate between bag cell neuron Ca$^{2+}$ currents (Gardam et al. 2008; Strong et al. 1987). Despite the lack of
selectivity of Ni$^{2+}$, we have found it to be an effective blocker that avoids problems like altering the Na$^{+}$/Ca$^{2+}$ exchanger or affecting K$^{+}$ channels, which have been reported for Cd$^{2+}$ and Co$^{2+}$, respectively (Agus et al. 1991; Le et al. 2005). This is important for experiments conducted under more physiological conditions, such as recording the prolonged depolarization in current-clamp. Moreover, Ni$^{2+}$ does not alter $I_{PD}$, making it useful to distinguish between cation and Ca$^{2+}$ currents.

The Ba$^{2+}$ permeability, modulation by PKC, and Ni$^{2+}$ block are consistent with the persistent Ca$^{2+}$ current being the same species of channel that makes up the rapid Ca$^{2+}$ current. How is it that a rapid current could generate a persistent current? There is a fair degree of overlap between the activation and inactivation curves for the rapid Ca$^{2+}$ current, which in principle provides an opportunity for a “window current”. Originally coined by Attwell et al. (1979) in reference to Na$^{+}$, the principle provides an opportunity for a “window current”. Originally coined by Attwell et al. (1979) in reference to Na$^{+}$, the window current is thought to be part of a fast, voltage-gated current that arises when the activation and inactivation processes coincide with respect to voltage. Within this voltage range window, the channel undergoes a continuous cycle of transitioning from open to inactivated to closed, followed by re-opening (Cohen and Lederer 1987; Reuter and Scholz 1977). That stated, while the persistent Ca$^{2+}$ current has a threshold between $-50$ and $-40$ mV, the amplitude of the prolonged depolarization can be as large as 15 mV but as small as 5 mV. This likely reflects a difference between the intracellular contents and uses nASW in the bath, and the less than physiological whole cell voltage-clamp, which replaces the intracellular contents with Cs$^{+}$-aspartate and uses Ca$^{2+}$-Cs$^{+}$-TEA ASW in the bath. Thus the current flow observed in bag cell neurons between $-50$ and $-20$ mV under voltage clamp is likely physiological and would influence the membrane potential under current clamp. This is reinforced by our Ca$^{2+}$-imaging showing that even small depolarizations are capable of evoking Ca$^{2+}$ influx in

FIG. 10. PKC activation enhances prolonged depolarization elicited by a current ramp mimicking $I_{PD}$. A: sample trace of prolonged depolarization evoked after a current ramp in a neuron pretreated with 0.1% DMSO (left) or a different neuron pretreated with 100 nM PMA for 20 min (right). The ramp is an inverted idealized version of $I_{PD}$. The peak depolarization evoked during current injection is similar in both cases; however, the PMA pretreatment results in a larger prolonged depolarization elicited after the injection is turned off. Scale bars apply to both panels. B: summary graph of mean peak depolarization elicited by the ramp current shows that the mean response in control vs. PMA is not significantly different (2-tailed unpaired Student’s t-test). C: summary graph of mean prolonged depolarization with and without PMA pretreatment. The difference between the means is significant (2-tailed unpaired Student’s t-test; Welch corrected).

FIG. 11. The prolonged depolarization is determined by dynamic interactions between different conductances. Schematic of bag cell neuron membrane potential during a prolonged depolarization. Ca$^{2+}$ entry via rapid, voltage-dependent Ca$^{2+}$ current occurs during the action potential train (step 1). This increase in intracellular Ca$^{2+}$ triggers a voltage-independent cation current that depolarizes the membrane and mediates the initial phase of the response. The positive change in membrane potential opens voltage-dependent persistent Ca$^{2+}$ current to maintain the prolonged depolarization even as the action current turns off (step 3).
normal extracellular medium, presumably by recruiting persistent Ca\(^{2+}\) current.

Typically, persistent Ca\(^{2+}\) current is ascribed to L-type Ca\(^{2+}\) channels, either characterized physiologically and/or pharmacologically in neurons and myocytes (Carlin et al. 2000a,b; Cohen and Lederer 1987; Fisher and Bourque 1995) or expressed in cell lines (McRory et al. 2004; Xu and Lipscombe 2001). In particular, Ca\(_V1.3\) and Ca\(_V1.4\) present activation thresholds that are within 10–20 mV of typical neuronal resting potentials and once open they inactivate very slowly. The extent that Ca\(^{2+}\)-dependent inactivation determines the steady-state amplitude of the bag cell neuron persistent Ca\(^{2+}\) current is unclear. Ca\(^{2+}\)-dependent inactivation in L-type channels is mediated by closely associated calmodulin (Zuhlke et al. 1999). This can be assessed by using Ba\(^{2+}\) as a charge carrier because it binds calmodulin poorly and lessens Ca\(^{2+}\)-dependent inactivation when passing through the channel (Chao et al. 1981; Tillotson 1979). However, similar to a report by McRory et al. (2004) on Ca\(_V1.4\), we found that the inactivation of both fast and persistent bag cell neuron Ca\(^{2+}\) currents is not dramatically slowed by Ba\(^{2+}\).

The bag cell neuron persistent Ca\(^{2+}\) current meets the criteria for a conductance that would act as a pacemaker current to maintain the membrane potential in an up-state, i.e., more positive than rest. However, to make this transition, the membrane potential must first be depolarized. Thus the Ca\(^{2+}\) current plays two roles in generating the prolonged depolarization: one, permitting substantial Ca\(^{2+}\) influx during the initial stimulus to activate the voltage-independent cation current (I\(_{\text{M}}\)) (Geiger and Magoski 2008; Hung and Magoski 2007); two, after the cation channel has brought the membrane potential into the threshold range of the Ca\(^{2+}\) current, the persistent mode then contributes steady-state inward current to promote the response (Fig. 11). In agreement with this, the duration of the depolarization is shortened when Ni\(^{2+}\) is introduced immediately after the train, whereas the magnitude of the response is suppressed when Ni\(^{2+}\) is applied once the prolonged depolarization is fully underway. Of course, we cannot rule out the possibility that some other current is a factor in the long-term effect on membrane potential. Given that the persistent current is by definition Ca\(^{2+}\) permeable, Ca\(^{2+}\) influx during the prolonged depolarization could trigger additional channels, including perhaps re-recruiting the cation current.

Neurons from the septal nucleus, stomatogastric ganglion, hippocampus, entorhinal cortex, Aplysia buccal ganglion, and lumbosacral spinal cord achieve activity-dependent change by employing Ca\(^{2+}\) current simply to deliver the requisite Ca\(^{2+}\) for cation channel opening (Dembrow et al. 2004; Derjean et al. 2005; Egorov et al. 2002; Fraser and MacVicar 1996; Hasuo et al. 1990; Zhang et al. 1995). Alternatively, dorsal horn, motor, Manduca, and suprachiasmatic neurons use persistent Ca\(^{2+}\) current exclusively for pacemaking (Carlin et al. 2000b; Kononenko and Dudek 2006; Lee and Heckman 1998; Mercer et al. 2005; Russo and Honsgaard 1996). There are two prior studies, concerning plateau potentials, with some similarities to our findings. Specifically, in subthalamic nucleus and dorsal horn neurons the initial phase of depolarization is due to persistent Ca\(^{2+}\) current, which in turn elicits a cation current that carries the latter phase (Beurrier et al. 1999; Morisset and Nagy 1999). For the bag cell neuron-prolonged depolarization, the interplay between Ca\(^{2+}\) and cation channel is a degree more sophisticated, requiring rapid Ca\(^{2+}\) current, then Ca\(^{2+}\)-activated cation current, followed by persistent Ca\(^{2+}\) current.

During an afterdischarge in the intact bag cell neuron cluster, the persistent current would contribute tonic inward current to maintain the neurons in a depolarized state necessary for action potential firing, the secretion of egg-laying hormone, and reproduction. The upregulation of PKC that occurs once the afterdischarge is underway (Wayne et al. 1999) could enhance both the rapid and persistent mode of the Ca\(^{2+}\) current, thus providing additional drive. Our work highlights how neurons in general may use interactions involving Ca\(^{2+}\) and cation channels to achieve long-term, activity-dependent changes in excitability with implications for the initiation of behavior.  

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