The Journal of Experimental Biology 201, 745–754 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JEB1257

CHARACTERIZATION OF Na^+ AND Ca^{2+} CURRENTS IN BAG CELLS OF SEXUALLY IMMATURE *APLYSIA CALIFORNICA*

LYNNE A. FIEBER*

Division of Marine Biology and Fisheries, University of Miami Rosenstiel School of Marine and Atmospheric Science, 4600 Rickenbacker Causeway, Miami, FL 33149-1098, USA

*e-mail: lfieber@rsmas.miami.edu

Accepted 15 December 1997: published on WWW 5 February 1998

Summary

The neurosecretory bag cells of sexually mature *Aplysia californica* release egg-laying hormones as part of the reproductive process after a train of action potentials termed afterdischarge. Whole-cell voltage-clamp experiments were performed in cultured cells from sexually immature *A. californica* to characterize the inward voltage-gated currents for Na^+ and Ca^{2+}. The goal of these experiments was to investigate the regulation of excitability during sexual maturation.

Na^+ currents in bag cells of immature *A. californica* were similar in several ways to those of mature animals. The Na^+ currents activated at voltages less negative than –30 mV and peaked at 10–20 mV in artificial sea water. The time course and pharmacology of bag cell Na^+ currents were similar to those of bag cells from mature *A. californica*, although the Na^+ current density was lower in immature *A. californica*. Na^+ currents were inhibited by tetrodotoxin (50 nmol l^{-1}). The Na^+ current was relatively insensitive to depolarized holding potentials (V_h), maintaining approximately 50 % of peak current amplitude present at V_h=–70 mV throughout the activation range at V_h=–30 mV. In experiments using a 1 s depolarized V_h prior to a test pulse, the half-inactivation voltage ($V_{1/2}$) was –27 mV. Recovery of immature Na^+ current from steady-state inactivation at V_h=–30 mV had a time constant (τ) of 9.5 ms, significantly slower than in mature animals.

Ca^{2+} currents of immature *A. californica* activated at approximately –30 mV and peaked at approximately 20 mV with 11 mmol l^{-1} Ba^{2+} as the charge carrier. The principle differences from mature Ca^{2+} currents were the low density of the immature Ca^{2+} currents and their 'run-down' in whole-cell recordings. The pharmacology and $V_{1/2}$ of bag cell Ca^{2+} currents were similar to those of L-type Ca^{2+} currents in mature cells. The Ca^{2+} currents were inhibited 61±10 % by nifedipine (10 μmol l^{-1}) and were unaffected by ω-conotoxin GVIA (10 μmol l^{-1}). The Ca^{2+} currents were relatively insensitive to depolarized V_h, activating maximally at V_h=–90, –70 and –50 mV, and maintaining 50 % of this peak current amplitude throughout the activation range at V_h=–30 mV. The $V_{1/2}$ was –23 mV in experiments in which cells were subjected to a 1 s depolarized V_h prior to a test pulse.

Na^+ current amplitudes were maintained or increased during 1 min of 4 Hz test pulses in bag cells at V_h=–70 mV and V_h=–30 mV. In contrast, Ca^{2+} current run-down occurred during 1 min of 4 Hz test pulses in seven of 10 cells at V_h=–70 mV and in 12 of 12 cells at V_h=–30 mV.

The observed scarcity of Na^+ and Ca^{2+} currents in immature bag cells as well as the specific characteristics of immature bag cell Ca^{2+} currents make repetitive action potential firing and hormone release less likely than in mature bag cells.

Key words: bag cell, patch-clamp, voltage-gated current, sexual immaturity, *Aplysia californica.*

Introduction

Sexual maturation in *A. californica* is defined by the onset of copulation and egg-laying behavior. Yet the reproductive system appears fully formed several months prior to sexual maturity when the animals enter stage 13 of development (stage 13; Cash and Carew, 1989). The appearance of the bag cell clusters on the abdominal ganglion is an important developmental feature of early stage 13, occurring at approximately 120 days post-metamorphosis. At this stage, the animals can be considered sexually immature adults.

The bag cells store hormones that cause egg laying when released. Both an 11 g sexually immature *A. californica* and a 200 g mature *A. californica* 4–6 months after reaching stage 13 are equipped to reproduce. Yet reproductive behavior and egg laying are observed only in *A. californica* weighing more than 150 g. The physiological features that distinguish 11 g from 200 g adult *A. californica* have been documented, leading to several hypotheses for the absence of reproductive behavior in *A. californica* weighing less than 150 g. Two possibilities are that sexually immature, early stage 13 animals do not synthesize bag cell hormones or that they do not release them. McAllister

et al. (1983) reported that, in early stage 13 *A. californica*, the first 10 cells of the newly forming abdominal ganglion contain messenger RNA for egg-laying hormone. Nick *et al.* (1996*b*) reported that bag cell clusters from immature *A. californica* (11–20 g) can be electrically stimulated to secrete egg-laying hormone in concentrations sufficient to induce egg-laying behavior in mature adults. These studies suggest that the pertinent hormones and the cellular machinery for secretion are present in immature *A. californica*. Nevertheless, an ability to release egg-laying hormones naturally may be lacking in sexually immature adults.

Hormone secretion from mature bag cells is caused by trains of action potentials called the afterdischarge (AD; Kupfermann and Kandel, 1970). Occasionally, a large electrical stimulus can provoke an AD in an excised bag cell cluster from stage 13 animals that are far from sexual maturity (11 g; Nick *et al.* 1996*a*). This is very rare, and the resulting AD is short-lived. The inability of young *A. californica* to sustain an AD coincided with high K^+ current densities and low Ca^{2+} current densities in immature *A. californica* compared with their sexually mature counterparts capable of AD.

The AD is caused in part by ion movement through channels that carry Na^+ and Ca^{2+} into the cell during an action potential. Characterization of the inward voltage-gated currents for Na^+ and Ca^{2+} in bag cells from sexually immature *A. californica* may reveal kinetic and pharmacological differences that provide insights into the regulation of excitability during sexual maturation. In a previous study (Fieber, 1995), Na^+ and Ca^{2+} currents of bag cells from *A. californica* of known sexual maturity were studied in short-term tissue culture. In the present study, bag cell Na^+ and Ca^{2+} currents from sexually immature *A. californica* were studied under exactly the same conditions.

Materials and methods

Aplysia californica were obtained from the University of Miami Aplysia Facility where they were raised from eggs laid by wild-caught brood stock and maintained in community tanks at 15 °C. All experiments were performed on cells from sexually immature animals. Sexual immaturity was defined by an absence of sexual behaviors such as copulation or egg laying. Subsequent observations on animals from the same cohorts as those used in experiments indicated that these cohorts did not engage in mating behavior for approximately 2 months after electrophysiological experiments.

Experiments to assess whole-cell ionic currents were performed with the whole-cell variation of the patch-clamp technique (Hamill *et al.* 1981) on cells maintained in short-term tissue culture at 19–21 °C. Abdominal ganglia with intact bag cell clusters from *A. californica* of known ages were removed from animals anesthetized for 30 min in a 1:1 mixture of isotonic $MgCl_2$ and sea water and were digested in an enzyme solution in artificial sea water (ASW) for 24 h at room temperature. The enzyme solution consisted of 12.5 mg of neutral protease (Boehringer Mannheim, Indianapolis, IN, USA), 5 mg of hyaluronidase and 1 mg of collagenase type XI (both from Sigma, St Louis, MO, USA) in 5 ml of ASW. The ASW consisted of (in mmol l^{-1}): 417 NaCl, 55 $MgCl_2$, 10 $CaCl_2$, 10 KCl, 10 Hepes/KOH, pH 7.6. After digestion, the connective tissue sheath was removed from the clusters and the bag cells were dispersed onto 35 mm culture dishes in ASW, plus 100 i.u. ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} fungizone, and maintained at room temperature (approximately 19–21 °C) until use in experiments 4–48 h later. Electrophysiological recordings were made with an Axopatch 200A patch-clamp amplifier, using the PClamp 6 programs (Axon Instruments, Foster City, CA, USA) for data acquisition and storage on a 100 MHz IBM-compatible computer. The intracellular solution consisted of (in mmol l^{-1}): 450 CsCl, 2.9 $CaCl_2$, 2.5 $MgCl_2$, 10 EGTA, 5 Na_2ATP, 300 μmol l^{-1} GTP and 40 Hepes/CsOH, pH 7.4. Pipette resistances were less than 1 MΩ. ASW was used for recording Na^+ and K^+ currents. Ca^{2+} currents were not recorded during Na^+ current recordings because of their relatively greater latency and small amplitude in 10 mmol l^{-1} Ca^{2+}. To study Ba^{2+} currents through Ca^{2+} channels, the solution consisted of Ba^{2+}-Cs^+-TEA^+ containing (in mmol l^{-1}): 460 tetraethylammonium chloride (TEACl), 10.4 CsCl, 11 $BaCl_2$, 55 $MgCl_2$ and 10 Hepes/CsOH, pH 7.6. The cells were bathed in different solutions during experiments by applying solutions from 1 μl pipettes attached to gravity-dispensed reservoirs. All voltage-activated currents were elicited from a holding voltage by test depolarizations with a duration of 25–500 ms separated by a pause of 4–10 s at the holding voltage.

Calculated voltage errors were usually smaller than 3 mV following approximately 80 % series resistance compensation. These errors were not corrected in the data presented. The PClamp 6 P/4 leak subtraction protocol was used in some of the data presented. Whole-cell capacitance was estimated from the capacitance setting on the patch-clamp amplifier.

All reagents were obtained from Sigma Chemical Co. unless otherwise noted. Tetrodotoxin (TTX; Research Biochemicals Inc.; Natick, MA, USA) was diluted and divided into small samples, then dried under N_2 and stored at −20 °C until it was dissolved in ASW at the time of experiments. Nifedipine and ω-conotoxin GVIA (ω-conotoxin) were obtained lyophilized from Calbiochem (San Diego, CA, USA). Nifedipine was diluted daily from stock solutions of 5 mmol l^{-1} in ethanol stored at −4 ° C in foil-wrapped vials, and was applied in the dark. Solutions of ω-conotoxin were made up at the time of use and were discarded after 24 h. ω-Conotoxin was applied to cells in a solution containing low levels of Ca^{2+} consisting of ASW solution lacking $CaCl_2$. After control recordings had been collected, each drug was bath-applied from a 1 μl pipette. Wash-out of drug effects using extracellular solution was attempted after studying the effects of the drug.

The Na^+ equilibrium potential was calculated from the Nernst equation using Na^+ activity values from Robinson and Stokes (1968). Data are presented as mean ± S.D. in the stated number of cells, unless otherwise noted.

Statistical comparisons were made with the Datadesk program (Data Description, Inc., Ithaca, NY, USA).

Results

Na^+ and Ca^{2+} currents are described from 54 bag cells from 24 *A. californica* from nine different egg masses. The animals studied ranged in age from 4.25 to 7 months and weighed 12–125 g. Cultured bag cells from immature animals were 10–30 μm in diameter. Input resistances were 1.19±1.7 GΩ (N=23). Less than 50 % of cells studied in whole-cell voltage-clamp experiments had inward Na^+ currents, Ca^{2+} currents or both, although outward currents were commonly recorded. The number of bag cells per culture with Na^+ and Ca^{2+} currents did not vary widely with the length of time cells were in culture.

Na$^+$ *currents*

Fig. 1 shows Na^+ currents in immature bag cells. Na^+ currents activated at test depolarizations less negative than −30 mV elicited from a holding potential (V_h) of −70 mV and peaked within 2 ms during each depolarizing test pulse (Fig. 1A). Na^+ current amplitude peaked at test depolarizations of 10–20 mV, as shown in the current–voltage relationship (*I–V*) of Fig. 1B. Although variable in amplitude, the Na^+ current densities of immature bag cells were approximately one-third of those of Na^+ currents in adult bag cells (approximately150 pA pF^{-1} at activation peak; Fieber, 1995). The average zero current potential was close to the Na^+ equilibrium potential of 84 mV.

Like mature Na^+ currents, immature Na^+ currents were susceptible to block by TTX (Fig. 2). In six bag cells from three different animals, Na^+ currents were reversibly blocked by 82±5 % by bath-applied 50 nmol l^{-1} TTX.

During an AD, mature bag cells undergo a change in resting potential to −30 mV (Fisher and Kaczmarek, 1990). The sensitivity of bag cell whole-cell Na^+ current to depolarized V_h was determined to assess the ability of Na^+ currents from immature bag cells to fire repetitively at −30 mV. Sensitivity to V_h was evaluated in three ways (Fig. 3). Families of Na^+ currents were recorded from different V_h values, Na^+ currents were recorded at a single test potential from different V_h values held for 1 s prior to the test pulse, and two repetitive depolarizations to a single test potential, with varied interval, were elicited from a V_h of −70 mV and −30 mV.

Changes in the duration of the depolarized V_h did not affect the amplitude of immature Na^+ currents. The *I–V* curves in Fig. 3A from an individual cell show that, when families of Na^+ currents were elicited from each V_h maintained for a total of approximately 1 min except during the depolarizations, the current amplitudes were maximal at a V_h of −70 and −50 mV, and were 54±7 % of maximum at a V_h of −30 mV (N=3). For comparison, a plot of the average Na^+ current amplitude as a function of V_h held for 1 s prior to a single test pulse is shown in Fig. 3B. The data were fitted to a Boltzman distribution with the voltage at which the current was half-inactivated ($V_{1/2}$) was calculated as −27 mV. This value of $V_{1/2}$ is similar to the degree of current inactivation observed when depolarized V_h was maintained for 1 min (Fig. 3A). The $V_{1/2}$ of Fig. 3B was not significantly different from the average mature adult $V_{1/2}$ for Na^+ current of −21 mV (Fieber, 1995; two-way analysis of

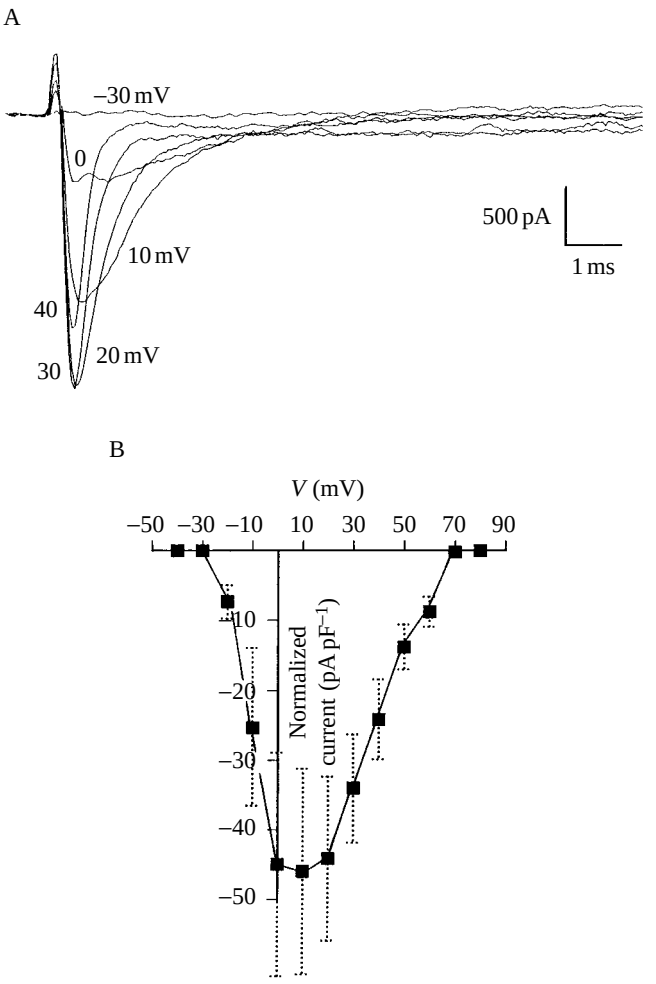

Fig. 1. Na^+ currents in cultured bag cells from immature *Aplysia californica*. Whole-cell voltage-clamp recordings in artificial sea water. (A) Na^+ currents at test potentials between −30 and 40 mV. Holding potential (V_h)=−70 mV. Test potentials lasted for 50 ms and occurred at 4 s intervals. (B) Average current–voltage (*I–V*) relationship for Na^+ current expressed as a function of whole-cell capacitance in 11 bag cells. Values are means ± S.E.M.

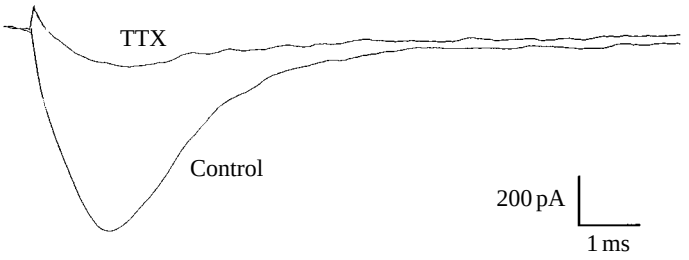

Fig. 2. Tetrodotoxin (TTX, 50 nmol l^{-1}) block of a Na^+ current in an immature bag cell. V_h=120 mV, test potential=10 mV. Test potentials were delivered at 4 s intervals.

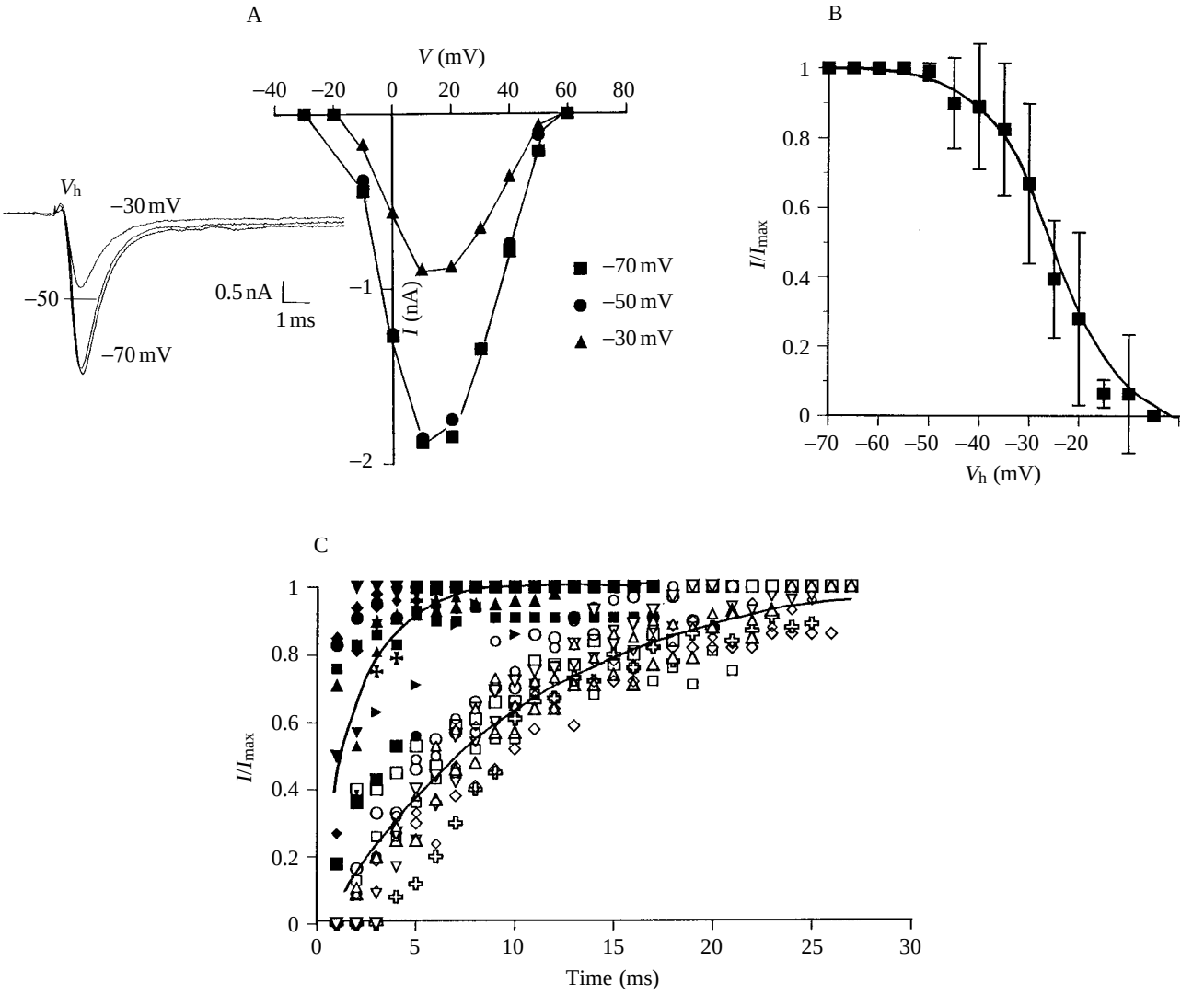

Fig. 3. Sensitivity of the Na^+ current to depolarized V_h and recovery from inactivation. (A) *I–V* curves recorded in a single cell at the indicated V_h. For clarity, only one curve is drawn for V_h=−70 mV and V_h=−50 mV. Inset: currents to 10 mV at the indicated V_h. (B) Plot of average normalized Na^+ current amplitude (I/I_{max}) in nine cells in artificial sea water at 10 mV at different V_h values maintained for 1 s prior to the test pulse. The curve was fitted to a Boltzman equation, $I/I_{max}=\{1+\exp[(V-V_{1/2})/s]\}^{-1}$, where s is the slope, $V_{1/2}$=−27 mV and s=6. (C) Recovery from inactivation in a double test pulse protocol. Filled symbols represent data collected at V_h=−70 mV, while open symbols represent data collected at V_h=−30 mV, with each cell designated by a unique symbol. Test potential=10 mV. Curves were fitted to $I/I_{max}=1-\exp(-x/\tau)$, where x is the time in ms and τ is the time constant. τ=1.9 ms at V_h=−70 mV (N=11) and τ=9.5 ms at V_h=−30 mV (N=10).

variance, ANOVA). Fig. 3C shows recovery of Na^+ currents from inactivation in a double-pulse protocol. Time constants were 1.9 ms at V_h=−70 mV, which is not significantly different from the adult time constant of 2.9 ms (Fieber, 1995; ANOVA), and 9.5 ms at V_h=−30 mV, which is significantly different from the adult value of 6.8 ms (two-way ANOVA, $P\leqslant 0.01$). Thus, Na^+ currents of immature bag cells were slower to recover from inactivation at V_h values relevant to the AD.

Ca^{2+} currents

Ca^{2+} currents, using Ba^{2+} as the charge carrier, were studied by eliciting test potentials from V_h=−70 mV. Ca^{2+} currents were short-lived in whole-cell recordings, beginning to run down within approximately 2 min of whole-cell dialysis and disappearing within 10 min of the whole-cell current recording configuration being established. Variations on the normal recording protocol were made in an attempt to inhibit Ca^{2+} current run-down. Maintaining the cells at a very negative V_h of −120 mV for 1 min before any test potentials to elicit Ca^{2+} currents did not inhibit run-down beginning within 2 min of whole-cell dialysis (N=3). Ca^{2+} currents also disappeared with time in cell-attached recordings of single Ca^{2+} channel currents in which the cytosol remained intact (data not shown).

Ca^{2+} currents were smaller in amplitude than Na^+ currents from the same cells. Ca^{2+} current densities in immature bag

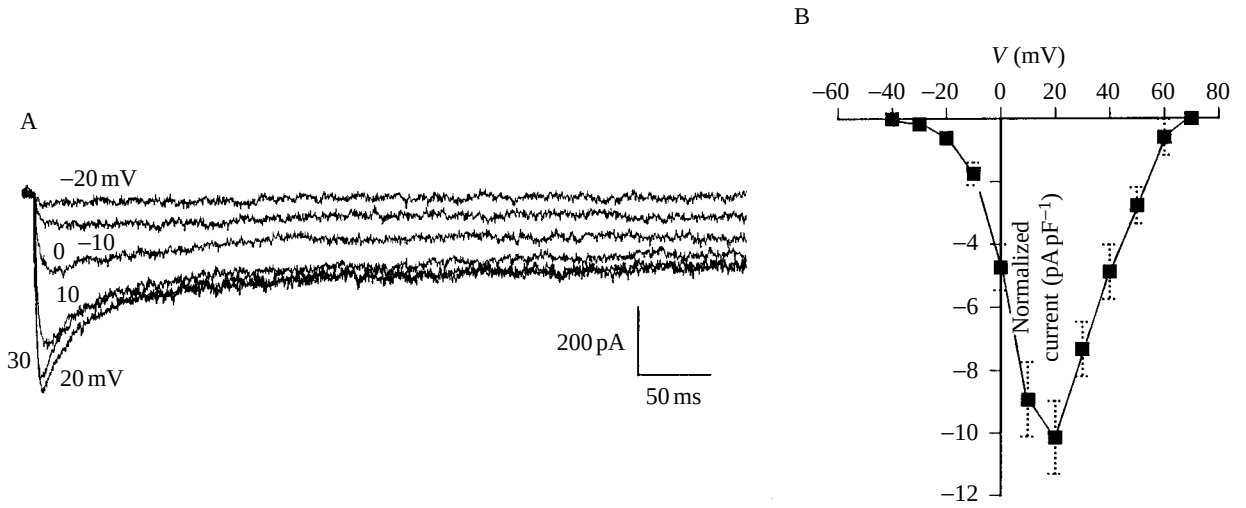

Fig. 4. Ca^{2+} currents in immature *Aplysia californica* bag cells. (A) Ca^{2+} currents recorded in Ba^{2+}-Cs^{+}-TEA^{+} at test potentials of −20 to 30 mV. V_h=−70 mV. Test potentials lasted for 400 ms and occurred at 6 s intervals. (B) Average current–voltage (*I–V*) relationship for Ca^{2+} currents expressed as a function of whole-cell capacitance in 23 bag cells. Values are means ± S.E.M.

cell were approximately 100 pA pF^{-1} at the activation peak, which is less than the Ca^{2+} current densities of mature bag cells (Fieber, 1995). Immature Ca^{2+} currents, like mature Ca^{2+} currents, activated at test potentials less negative than approximately −40 mV and peaked at approximately 20 mV (Fig. 4A,B). Immature Ca^{2+} currents were blocked by the Ca^{2+} channel blocker Cd^{2+} (100 μmol l^{-1}; not shown; Bean, 1989).

The pharmacological sensitivity of Ca^{2+} currents, with examples shown in Fig. 5, was similar to that of L-type currents in mature bag cells. Blocking using bath-applied nifedipine (10 μmol l^{-1}) was variable, ranging from 0 to 100 % block with an average block of 61±10 % in 11 bag cells (Fig. 5A). Block by ω-conotoxin (10 μmol l^{-1}) was ⩽5 % in six cells, with an average block of 2.3±5 % (Fig. 5B).

Fig. 6 shows the susceptibility of peak Ca^{2+} current amplitude to depolarized V_h. Like immature Na^{+} currents, immature Ca^{2+} current amplitudes were not affected by changing the duration for which the depolarized V_h was maintained. The *I–V* curves recorded at different V_h values maintained for approximately 1 min (Fig. 6A) show that peak current declined at a V_h of −30 mV. The plot of different V_h values maintained for 1 s before each test pulse *versus* whole-cell Ca^{2+} current amplitude is shown in Fig. 6B. These data are fitted by a Boltzman distribution with a $V_{1/2}$ of −23 mV; the mature adult average $V_{1/2}$ was −34 mV (Fieber, 1995). These values are not significantly different (two-way ANOVA). A depolarized $V_{1/2}$ is characteristic of L-type Ca^{2+} currents.

Immature bag cells may be incapable of action potential trains because their channels are regulated in an inactivated state. To test for the presence of inactivated Ca^{2+} channels capable of being opened by a depolarizing prepulse (Artalejo *et al.* 1991*a*,*b*), experiments were performed on nine cells with Ca^{2+} currents in which 5 ms prepulses to 120 mV were given immediately prior to each of 20 successive test pulses to elicit Ca^{2+} currents. For comparison, five cells with Ca^{2+} currents were subjected to test pulses without the 120 mV prepulse. Test pulses were to 20 mV from a V_h of −90 mV. All test pulses were separated by 10 s at −90 mV. Of the nine cells in which the 120 mV prepulse protocol was used, the initial Ca^{2+} current amplitude increased by 20–60 % during successive test pulses in five cells, was maintained in three cells, and decreased in one cell. Of the five cells with Ca^{2+} currents not receiving the 120 mV prepulse, the initial Ca^{2+} current amplitude was

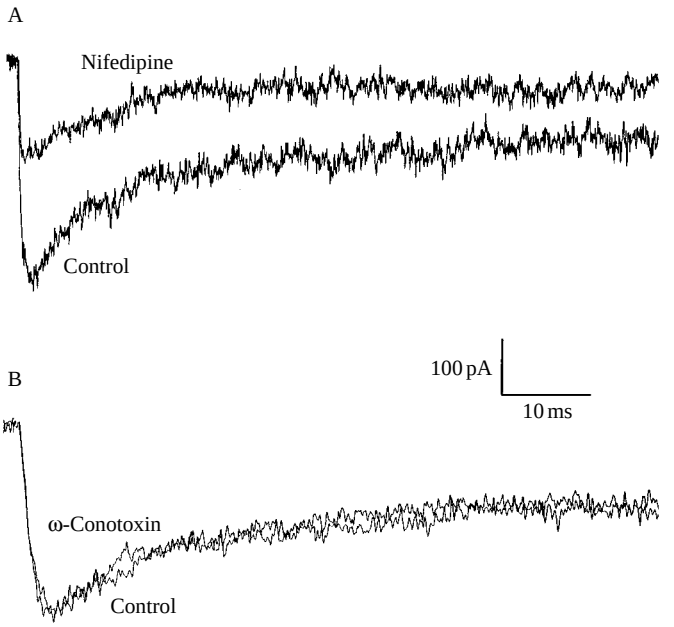

Fig. 5. Pharmacology of Ca^{2+} currents in immature bag cells. (A) Nifedipine (10 μmol l^{-1}) block of a Ca^{2+} current. V_h=−70 mV, test potential=20 mV. Test potentials were delivered at 6 s intervals. (B) Lack of inhibition by ω-conotoxin (10 μmol l^{-1}). V_h=−70 mV, test potential=20 mV. Test potentials were delivered at 6 s intervals.

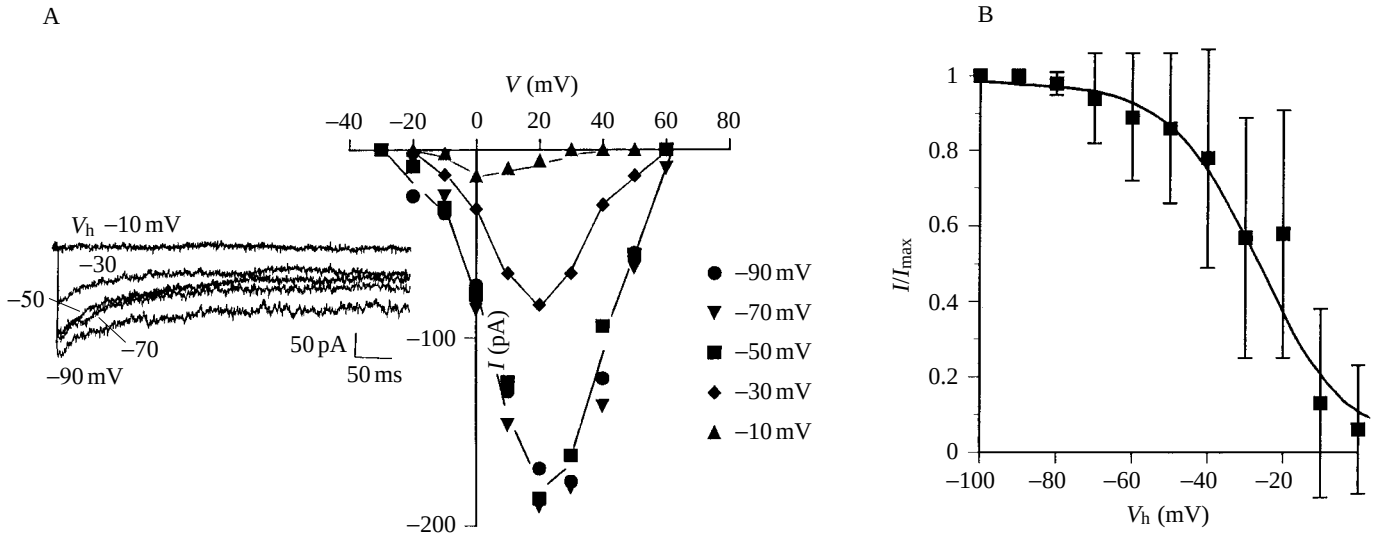

Fig. 6. Sensitivity of the Ca^{2+} current to depolarized V_h. (A) *I–V* curves recorded in a single cell at the indicated V_h. Inset: currents to 20 mV at the indicated V_h. (B) Plot of average normalized Ca^{2+} current amplitude (I/I_{max}) at 20 mV in Ba^{2+}-Cs^+-TEA^+ in 14 cells at different V_h maintained for 1 s prior to the test pulse. The curve was fitted to a Boltzman equation with $V_{1/2}$=−23 mV, *s*=11 (see Fig. 3). Values are means ± S.E.M.

maintained in three cells, increased by 20 % in one cell and decreased in one cell. In the eight cells in which the 120 mV prepulse preserved or enhanced Ca^{2+} current amplitudes during the 20 test pulses, however, the Ca^{2+} currents were irretrievably less than 50 % of the starting amplitude within 12 min of whole-cell dialysis.

Na^+ and Ca^{2+} currents during 4 Hz stimulation

The slower recovery of immature bag cell Na^+ currents from inactivation and the labile nature of the immature bag cell Ca^{2+} currents compared with mature *A. californica* bag cells suggest that immature currents might not be sustained at the action potential frequencies observed during an AD. To test this possibility, Na^+ and Ca^{2+} currents were elicited for 10 and 20 ms, respectively, at 4 Hz, an action potential frequency typical of the initial stage of AD (Dudek and Kossatz, 1982). V_h in these experiments was −70 mV or −30 mV, values that are typical of the initial, Na^+-dependent phase (−70 mV) and the later, Ca^{2+}-dependent phase (−30 mV) of the AD, respectively (Fisher and Kaczmarek, 1990). The results are shown in Fig. 7 for Na^+ currents and in Fig. 8 for Ca^{2+} currents. 4 Hz Ca^{2+} current experiments were executed within 2 min of the start of whole-cell dialysis, a period during which little Ca^{2+} current run-down occurred in other whole-cell Ca^{2+} current experiments.

Na^+ current amplitudes were maintained or increased during 1 min of 4 Hz test pulses in three cells at a V_h of −70 mV (Fig. 7A). Na^+ currents were also maintained or increased at a V_h of −30 mV (Fig. 7B) in three out of four experiments. In contrast, Ca^{2+} current run-down during the 1 min protocol was common, occurring in seven out of 10 cells at a V_h of −70 mV (Fig. 8A) and in 12 out of 12 cells at a V_h of −30 mV (Fig. 8B).

Discussion

Activation of voltage-gated Na^+, Ca^{2+} and K^+ currents by an initial electrical or chemical stimulus causes 30 min of repetitive action potentials in *A. californica* bag cells, known as the afterdischarge (AD). This AD causes hormone release from bag cells in sexually mature animals (Kupfermann and Kandel, 1970). In contrast, immature bag cells from animals weighing less than 11 g cannot sustain a full AD when they are exogenously stimulated (Nick *et al.* 1996*a*), and thus they do not exhibit AD-dependent hormone release. In the latter study, the ability to sustain an AD correlated with increasing animal mass between 11 and 50 g. The development of the capability of sexually immature *A. californica* to sustain an AD coincided with a decrease in the current density of three outward K^+ currents and an increase in the current density of two Ca^{2+} currents and the A-type K^+ current. The present study suggests several reasons additional to the observations of these authors why the bag cell clusters from a percentage of immature *A. californica* failed to sustain an AD. These additional reasons are a low percentage of cells with Na^+ and Ca^{2+} currents, a low density of Na^+ currents, and the time- and voltage-dependent disappearance of Ca^{2+} currents in whole-cell recordings.

The Na^+ and Ca^{2+} currents of mature *A. californica* bag cells have been well characterized (DeReimer *et al.* 1985*b*; Fieber, 1995; Nerbonne and Gurney, 1987; Strong *et al.* 1987). Ionic currents in bag cells of sexually immature *A. californica* studied in short-term tissue culture had many similarities to those present in bag cells from sexually mature *A. californica* under the same conditions. The activation range, time course, pharmacology and susceptibility to depolarized V_h of immature Na^+ and Ca^{2+} currents are all nearly identical to these characteristics in mature bag cell currents. The similarities are sufficiently numerous to suggest that the Na^+ and Ca^{2+}

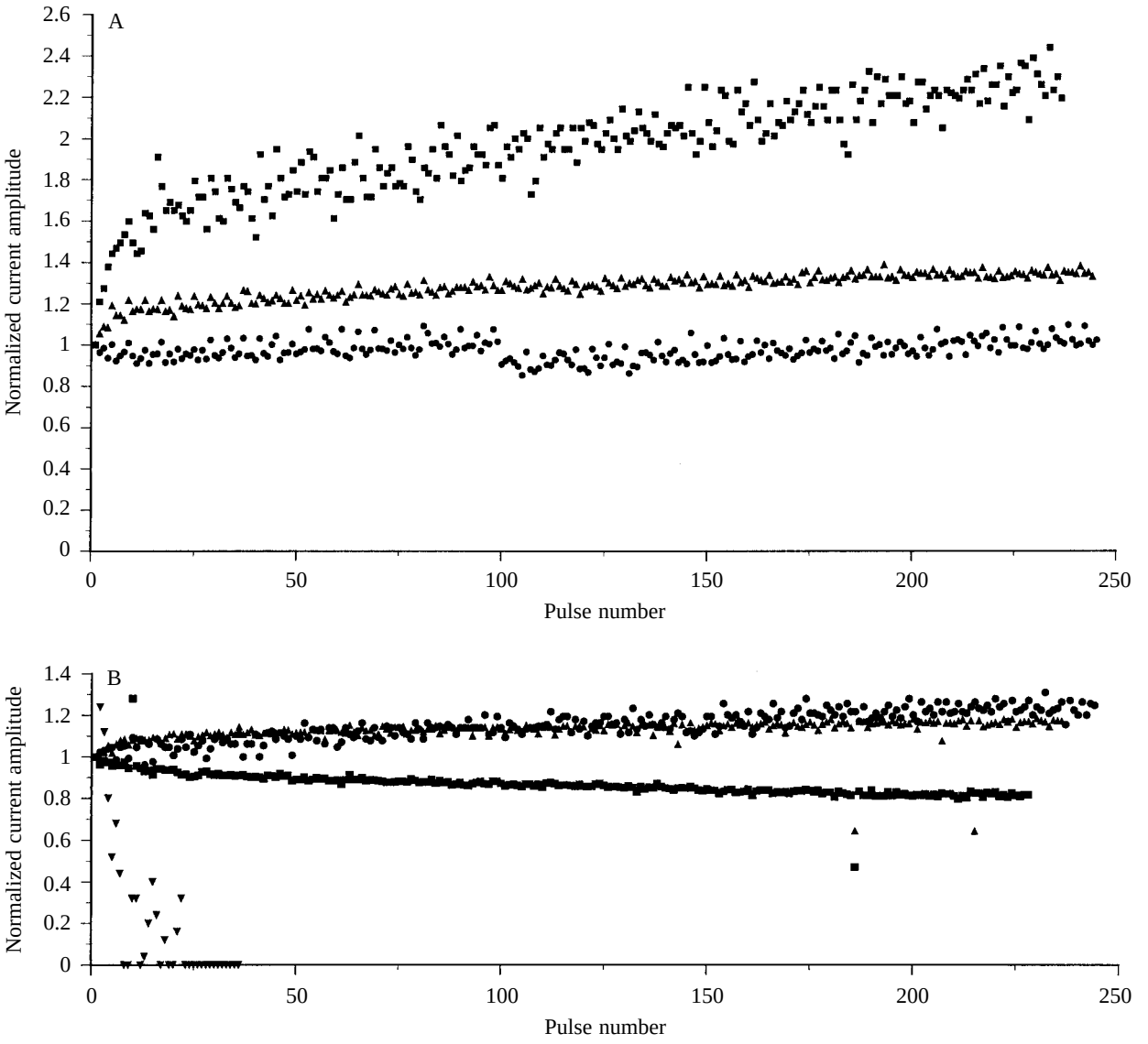

Fig. 7. Na^+ currents during depolarizations delivered at 4 Hz. (A) Na^+ currents in three bag cells in which currents were elicited by depolarizations to 10 mV for 10 ms from a V_h of −70 mV. Depolarization frequency was 4 Hz. Each symbol represents a unique cell. (B) Na^+ currents in four bag cells under the same conditions as in A but from a V_h of −30 mV.

channels are the same molecular entities in immature and mature *A. californica*. The failure of immature bag cells to sustain an AD may be due in part to the relatively lower abundance of functional Na^+ and Ca^{2+} channels in immature *A. californica* and the lability of immature bag cell Ca^{2+} currents.

Only 50 % of cultured bag cells from immature *A. californica* ranging from 4.25 to 7 months in age at 12–125 g had measurable inward currents, in contrast to 90 % of bag cells from mature animals cultured under the same conditions (Fieber, 1995). Nick *et al.* (1996*a*) found that immature bag cells from animals incapable of an AD at body masses less than 11 g responded to TEA^+ or to electrical stimulation with long depolarizations that sometimes summated to an action potential. Nick *et al.* (1996*a*) did not report the percentage of immature cells per *A. californica* cohort that responded with a depolarization, indicating at least the presence of Ca^{2+} currents and K^+ currents, or the percentage that had whole-cell Ca^{2+} currents. The generally lower Ca^{2+} current density of immature bag cells that probably contributed to the failure to sustain an AD in some immature animals was also observed by these authors. New observations made here, such as a similar, low density of Na^+ currents and differences in the number of immature bag cells possessing functional Na^+ and Ca^{2+} currents, might further explain the inhibition of the AD in a percentage of immature animals.

Nick *et al.* (1996*a*) observed that, as the mass of the animal increased above 11 g, an increasing proportion of bag cell clusters were capable of AD and that excised bag cell clusters from *A. californica* over 50 g were capable of producing an

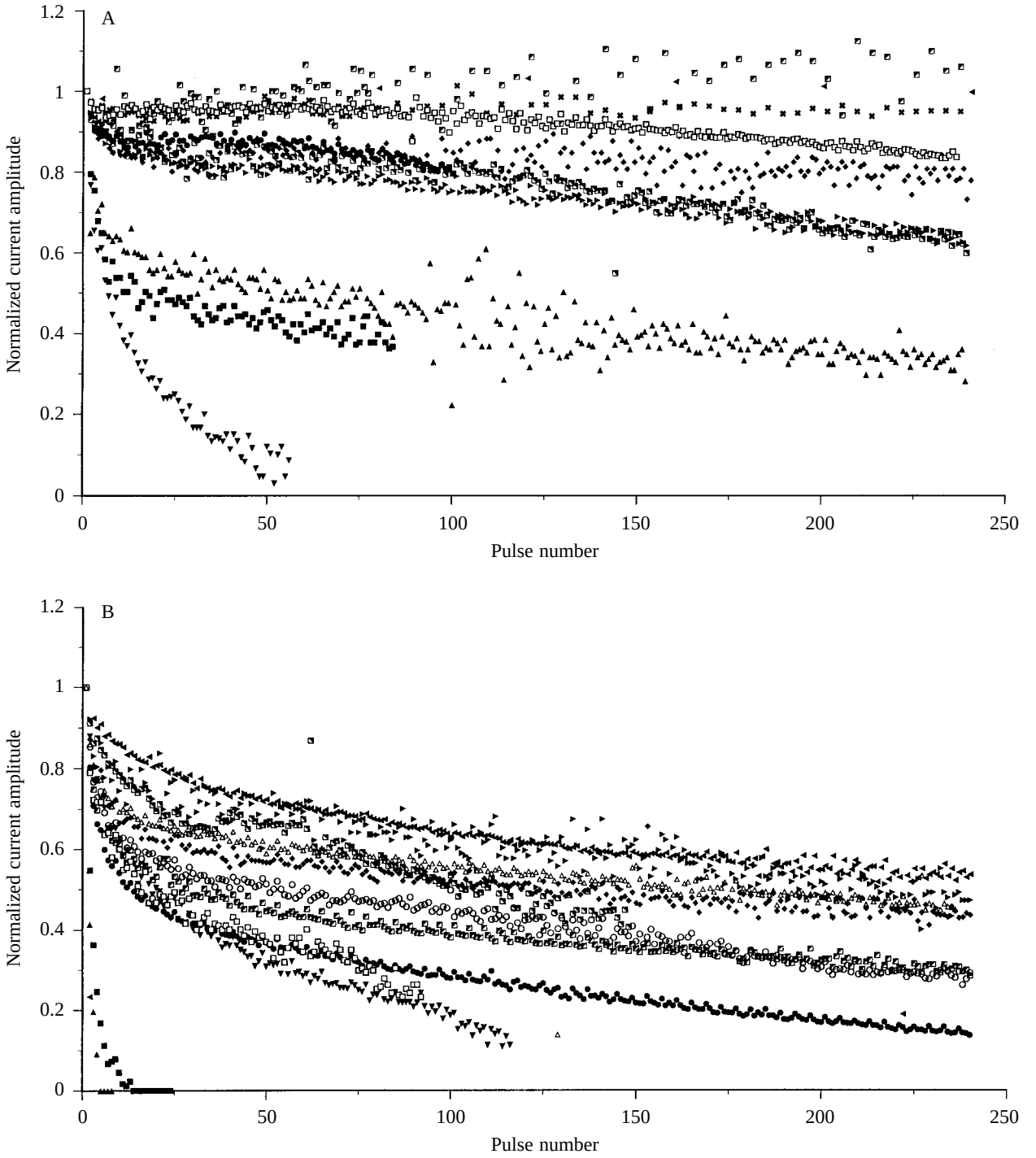

Fig. 8. Ca^{2+} currents during depolarizations delivered at 4 Hz. (A) Ca^{2+} currents in 12 bag cells in which currents were elicited by depolarizations to 20 mV for 20 ms from a V_h of −70 mV. Depolarization frequency was 4 Hz. Each symbol represents a unique cell. (B) Ca^{2+} currents in 12 bag cells under the same conditions as in A but from a V_h of −30 mV. Each symbol represents a unique cell. These cells are the same as those in A.

AD. In the present study, the proportion of dissociated bag cells with measurable Na^+ and Ca^{2+} currents in this exact size range was uniformly low. There are several explanations for why an AD might occur in some 11–50 g *A. californica* despite the scarcity of Na^+ and Ca^{2+} currents observed in cultured bag cells from animals in this size range. First, there may be abundant bag cells in clusters from 11–50 g animals with Na^+ and Ca^{2+} channels, but these cells rarely survive in culture. This explanation would require a large difference in the survivorship of bag cells from immature and mature *A. californica* in culture, which is not likely since bag cell cultures of mature (Fieber, 1995) and immature *A. californica* yield

approximately the same number of cells after enzymatic treatment. Second, bag cells from 11–50 g animals may lose functional channels in culture. This explanation is also unlikely because approximately 50 % of the bag cells from immature animals dissociated only 4 h before experimentation lack functional Na^+ and Ca^{2+} channels, and this number does not vary widely with the time that cells are in culture, as would be expected if channels were being resynthesized, degraded or downregulated in large numbers. Third, low Na^+ and Ca^{2+} channel densities in cultured bag cells from 11–50 g *A. californica* may be representative of channel numbers *in vivo*, but these bag cell clusters may require very few electrically excitable cells to sustain at least a short AD. The high input resistances of immature compared with mature bag cells (Nick *et al.* 1996*a*; present study) suggest that relatively fewer Na^+ and Ca^{2+} channels would be required in immature cells than in mature cells for the generation of an action potential. However, the minimum number of action-potential-capable cells in a cluster required to sustain an AD is unknown.

The 4 Hz test pulse experiments suggest that Na^+ currents do not inactivate with 4 Hz activation but that Ca^{2+} currents do inactivate. These experiments were conducted under conditions in which AD-induced second messengers known to increase Na^+ and Ca^{2+} currents and to sustain the AD in mature *A. californica* (Kaczmarek and Strumwasser, 1984; DeReimer *et al.* 1985*b*; Fieber, 1995) are absent. The availability of Na^+ currents to be sustained during 4 Hz stimulation suggests that bag cells of immature *A. californica* are probably capable of repetitive firing during the initial, 1 min Na^+-dependent phase of the AD (Kaczmarek *et al.* 1982) in spite of their greater tendency to inactivate in a standard double-pulse protocol than mature Na^+ currents. In contrast, Ca^{2+} currents in bag cells of a percentage of immature *A. californica* may not be available during the subsequent, Ca^{2+}-dependent phase of the AD. Protein kinase C levels increase during the AD to enhance Ca^{2+} current amplitudes. It is not known whether the manoeuvres Nick *et al.* (1996*a*) used to initiate depolarizations in immature bag cells (TEA^+, 0.1 nA current injection) increase protein kinase C levels. These authors demonstrated that protein-kinase-C-sensitive Ca^{2+} currents are not present in immature *A. californica* under 11 g, suggesting that protein kinase C does not play the same role in maintenance of immature Ca^{2+} currents as it does in mature Ca^{2+} currents. It is possible that protein kinase C may prevent the Ca^{2+} current run-down observed in immature bag cells.

The run-down of Ca^{2+} currents in immature bag cells indicates that these currents may be susceptible to wash-out, use-dependent block or cumulative inactivation. Experiments to determine the contribution of whole-cell dialysis *versus* use-dependent inhibition to Ca^{2+} current run-down were inconclusive. Short-term (4 min) run-down was prevented in some cells by using depolarizing prepulses to avoid use-dependent block or cumulative inactivation, but this procedure did not prevent eventual Ca^{2+} current run-down during the course of longer experiments. Holding the cells at a negative V_h for 1 min before test potentials to elicit currents did not retard subsequent Ca^{2+} current run-down. This is an additional indication that run-down was not solely activity-dependent. However, the failure to avoid run-down in cell-attached single-channel experiments indicates that whole-cell dialysis is not the sole cause of run-down. Since Ca^{2+} current run-down was not observed in mature bag cells *in vitro* (Fieber, 1995), it is unlikely that the current carrier, Ba^{2+}, or the constituents of the intracellular solution are overtly damaging to bag cells. The possibility remains that immature bag cells *in vivo* have labile Ca^{2+} currents during repetitive activation of this current. Although Ca^{2+} current run-down and cumulative inactivation are observations that support the idea of resting modulation to prevent the AD, both occurred during studies of isolated Ca^{2+} currents in the absence of possible modulating effects of depolarizations that activate K^+ currents. Action potentials themselves are stimulatory in mature bag cells.

Modulation of Na^+ and Ca^{2+} currents by cyclic AMP and protein kinase C changes the height, duration and frequency of action potentials during the AD in sexually mature *A. californica* (Kaczmarek *et al.* 1978, 1980; Kaczmarek and Strumwasser, 1981, 1984; DeReimer *et al.* 1985*a*,*b*; Strong *et al.* 1987), sustaining and strengthening it. The scarcity of Na^+ and Ca^{2+} currents in whole-cell recordings from immature *A. californica* and the run-down of Ca^{2+} currents, together with the up-regulation of K^+ currents in immature *A. californica* observed by Nick *et al.* (1996*a*), suggest that bag cell currents are also regulated in sexually immature adults, but with a different result from that in mature adults. Ion channel modulation in immature bag cells may retard the occurrence of action potentials rather than causing changes in action potential amplitude and frequency, as occurs in mature adults. Such regulation would be present in closed or resting channels of immature *A. californica* and regulation would retard the opening of Na^+ and Ca^{2+} channels in response to a voltage stimulus that would otherwise open them, and would ensure that outward K^+ flow during depolarizations inhibits action potentials and the premature release of egg-laying hormones. This form of regulation could be effected by phosphorylation of ion channels which, in the case of bag cell Ca^{2+} channels, is known to be associated with changes in their functional expression during the AD (Fisher and Kaczmarek, 1990; DeReimer *et al.* 1994).

The trigger for a change from the resting state may be a hormonal or electrical signal, transforming functionally inexcitable cells into cells that are able to contribute to repetitive action potential firing within the bag cell cluster.

Supported by NSF IBN-9508705 and NIH RR-10294. The NCRR supports investigator-initiated research grants for the study of various non-mammalian systems. Please contact Dr Jill Carrington at gillc@ep.ncrr.nih.gov or (301) 435-0776 for more information.

References

ARTALEJO, C. R., DAHMER, M. K., PERLMAN, R. L. AND FOX, A. P.

(1991*a*). Two types of Ca^{2+} currents are found in bovine chromaffin cells: facilitation is due to the recruitment of one type. *J. Physiol., Lond.* **432**, 681–707.

ARTALEJO, C. R., MOGUL, D. J., PERLMAN, R. L. AND FOX, A. P. (1991*b*). Three types of bovine chromaffin cell Ca^{2+} channels: facilitation increases the opening probability of a 27 pS channel. *J. Physiol., Lond.* **444**, 213–240.

BEAN, B. P. (1989). Classes of calcium channels in vertebrate cells. *A. Rev. Physiol.* **51**, 367–384.

CASH, D. AND CAREW, T. J. (1989). A quantitative analysis of the development of the central nervous system in juvenile *Aplysia californica*. *J. Neurobiol*. **20**, 25–47.

DEREIMER, S. A., GREENGARD, P. AND KACZMAREK, L. K. (1985*a*). Calcium/phosphatidylserine/diacylglycerol-dependent protein phosphorylation in the *Aplysia californica* nervous system. *J. Neurosci.* **5**, 2672–2676.

DEREIMER, S. A., KACZMAREK, L. K., LAI, Y., MCGUINNES, T. L. AND GREENGARD, P. (1984). Calcium/calmodulin-dependent protein phosphorylation in the nervous system of *Aplysia*. *J. Neurosci*. **4**, 1618–1625.

DEREIMER, S. A., STRONG, J. A., ALBERT, K. A., GREENGARD, P. AND KACZMAREK, L. K. (1985*b*). Enhancement of calcium current in *Aplysia californica* neurones by phorbol ester and protein kinase C. *Nature* **313**, 313–316.

DUDEK, F. E. AND KOSSATZ, A. (1982). Conduction velocity and spike duration during afterdischarge in neuroendocrine bag cells of *Aplysia*. *J. Neurobiol.* **13**, 319–326.

FENWICK, E., MARTY, A. AND NEHER, E. (1982). Sodium and calcium channels in adrenal chromaffin cells. *J. Physiol., Lond*. **416**, 421–434.

FIEBER, L. A. (1995). Characterization of Na^+ and Ca^{2+} currents underlying the action potential in bag cells of *Aplysia*. *J. exp. Biol*. **198**, 2337–2347.

FISHER, T. E. AND KACZMAREK, L. K. (1990). The regulation of excitability by second messengers and protein kinases in peptidergic neurons of *Aplysia*. *Adv. second Messenger Phosphorylation Res.* **24**, 41–44.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. AND SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* **391**, 85–100.

JENNINGS, K. R., KACZMAREK, L. K., HEWICK, R. M., DREYER, W. J. AND STRUMWASSER, F. (1982). Protein phosphorylation during afterdischarge in peptidergic neurons of *Aplysia*. *J. Neurosci.* **2**, 158–168.

KACZMAREK, L. K., JENNINGS, K. AND STRUMWASSER, F. (1978). Neurotransmitter modulation, phosphodiesterase inhibitor effects and cyclic AMP correlates of afterdischarge in peptidergic neurites. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5200–5204.

KACZMAREK, L. K., JENNINGS, K. R., STRUMWASSER, F., NAIRN, A. C., WALTER, U., WILSON, F. D. AND GREENGARD, P. (1980). Microinjection of catalytic subunit of cyclic AMP-dependent protein kinase enhances calcium action potentials of bag cell neurons in cell culture. *Proc. natn. Acad. Sci. U.S.A.* **77**, 7487–7491.

KACZMAREK, L. K. AND STRUMWASSER, F. (1981). The expression of long lasting afterdischarge by isolated *Aplysia californica* bag cell neurons. *J. Neurosci*. **1**, 626–634.

KACZMAREK, L. K. AND STRUMWASSER, F. (1984). A voltage-clamp analysis of currents underlying cyclic AMP-induced membrane modulation in isolated peptidergic neurons of *Aplysia californica*. *J. Neurophysiol*. **52**, 340–349.

KUPFERMANN, I. AND KANDEL, E. R. (1970). Electrophysiological properties and functional interconnections of two symmetrical neurosecretory clusters (bag cells) in abdominal ganglion of *Aplysia californica*. *J. Neurophysiol*. **33**, 865–876.

MCALLISTER, L. B. R. J., SCHELLER, E. R. AND AXEL, R. (1983). *In situ* hybridization to study the origin and fate of identified neurons. *Science* **222**, 800–808.

NERBONNE, J. M. AND GURNEY, A. M. (1987). Blockade of Ca^{2+} and K^+ currents in bag cell neurons of *Aplysia californica* by dihydropyridine Ca^{2+} antagonists. *J. Neurosci*. **7**, 882–893.

NICK, T. A., KACZMAREK, L. K. AND CAREW, T. J. (1996*a*). Ionic currents underlying developmental regulation of repetitive firing in*Aplysia* bag cell neurons. *J. Neurosci.* **16**, 7583–7598.

NICK, T. A., MORIERA, J. E., KACZMAREK, L. K., CAREW, T. J. AND WAYNE, N. L. (1996*b*). Developmental dissociation of excitability and secretory ability in *Aplysia* bag cell neurons. *J. Neurophysiol*. **76**, 3351–3369.

ROBINSON, R. A. AND STOKES, R. H. (1968). *Electrolyte Solutions*, 2nd edn. London: Butterworths Pub.

STRONG, J. A., FOX, A. P., TSIEN, R. W. AND KACZMAREK, L. K. (1987). Stimulation of protein kinase C recruits covert calcium channels in *Aplysia* bag cell neurons. *Nature* **325**, 714–717.