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CHARACTERIZATION AND MODULATION OF Na^+ AND Ca^{2+} CURRENTS UNDERLYING THE ACTION POTENTIAL IN BAG CELLS OF TWO SPECIES OF *APLYSIA*

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

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Summary

The neurosecretory bag cells of *Aplysia* produce long trains of action potentials (afterdischarge) to release hormones important to egg laying. These ionic currents are modulated by second messengers. Modulation of excitability in bag cells is incompletely understood partly because the currents that are modulated have not been fully characterized. Whole-cell voltage-clamp experiments were executed in cultured cells from sexually mature *A. californica* and *A. brasiliana* to characterize the inward voltage-gated currents for Na^+ and Ca^{2+}. These species had similar Na^+ and Ca^{2+} current characteristics. The Na^+ currents activated at voltages less negative than −30 mV and peaked at between +10 and +20 mV in artificial sea water. The time course and pharmacology of bag cell Na^+ currents were similar to those of fast Na^+ current in other excitable cells. Na^+ currents were abolished in Na^+-free extracellular solution and were not inhibited by Cd^{2+}. The K_D for inhibition by tetrodotoxin was 2.6 nmol l^{-1}. The Na^+ current was relatively insensitive to depolarized holding potentials (V_h), maintaining approximately 65 % of peak current amplitude 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 −21 mV. The time constant of recovery from steady-state activation was 2.9 ms at V_h=−70 mV and 6.8 ms at V_h=−30 mV. The Ca^{2+} currents activated near −10 mV and peaked at approximately +20 mV with 11 mmol l^{-1} Ba^{2+} as the charge carrier. The pharmacology and $V_{1/2}$ of bag cell Ca^{2+} current were similar to those of L-type Ca^{2+} currents. In extracellular solution without Na^+, but containing Ba^{2+}, Cs^+ and tetraethylammonium, the Ca^{2+} current was inhibited by 25–100 % by nifedipine (10 μmol l^{-1}), mean 42 %, and was unaffected in the majority of cells by ω-conotoxin (10 μmol l^{-1}). The Ca^{2+} current was insensitive to Ni^{2+} (100 μmol l^{-1}), but was abolished by 100 μmol l^{-1} Cd^{2+}. Like the Na^+ current, the Ca^{2+} current was relatively insensitive to depolarized V_h, maintaining more than 80 % of peak current amplitude throughout the activation range at V_h=−40 mV. With a 1 s depolarized V_h prior to a test pulse, the $V_{1/2}$ was −30 mV. The activation thresholds for Na^+ and Ca^{2+} currents as well as the relative insensitivity of both currents to depolarized V_h as low as −30 mV are characteristics that would be required for spontaneous depolarizations during afterdischarge. After a 1 h treatment with phorbol 12-myristate 13-acetate (25 nmol l^{-1}), the amplitude of Na^+, Ca^{2+} and outward currents in individual bag cells was increased compared with control amplitudes from the same cells. Phorbol ester treatment did not change the activation and inactivation ranges, time course, $V_{1/2}$ or pharmacology of the inward currents.

Key words: bag cells, patch clamp, voltage-gated current, *Aplysia californica*, *Aplysia brasiliana*.

Introduction

The bag cells of the marine opisthobranch mollusc *Aplysia* are a homogeneous population of peptidergic neurosecretory cells in the central nervous system (CNS) that undergo prolonged changes in excitability that serve the purpose of releasing the hormones that initiate and control egg laying (Kupferman and Kandel, 1970). Secretion from bag cells is initiated by a period of synchronized firing involving all cells of the bilaterally distributed ganglia. This prolonged electrical discharge, called afterdischarge, initiates in the pleurovisceral connectives linking the abdominal ganglia with the head ganglia, the only afferent pathway to the bag cells (Pinsker and Dudek, 1977).

The afterdischarge consists of two phases of enhanced excitability that, together, last about 30 min and involve the voltage-gated ion channels characteristic of all excitable cells. Bag cells have both Na^+ channels and Ca^{2+} channels as the

conduits for inward currents, although Na^+ currents were reported as rare in voltage-clamp recordings (Kaczmarek and Strumwasser, 1984). Evidence that they contribute to excitability and to repetitive firing during afterdischarge comes from the observation that the first phase of afterdischarge consists of action potentials that depend on Na^+, but not Ca^{2+}, in the extracellular solution. Similarly, the second phase of afterdischarge is dependent on extracellular Ca^{2+}, is characterized by action potentials of longer duration than are seen in the first phase (Dudek and Kossatz, 1982) and can be blocked by Co^{2+}, which is known to block Ca^{2+} channels.

The afterdischarge, although dependent on an initiating stimulus in the connective into which bag cell processes extend, can proceed independently once initiated, even in cultured cells (Kaczmarek *et al.* 1979). The excitability of bag cells is regulated through two second messenger systems: adenyl cyclase, which decreases K^+ currents, and phospholipase C, which increases Ca^{2+} currents (DeReimer *et al.* 1985*b*; Kaczmarek *et al.* 1980; Kaczmarek and Strumwasser, 1981; Strong *et al.* 1987). Both types of modulation lead to changes in the shape of the action potential and affect action potential production during afterdischarge.

Although many details are known about ionic currents operating during afterdischarge and their modulation, which contributes to the initiation and cessation of afterdischarge, these details do not fully characterize the phenomenon. Basic kinetic and pharmacological data on some of these ionic currents are incomplete, making inferences about some aspects of their modulation difficult to interpret. K^+ currents and their modulation have been extensively studied (Kaczmarek *et al.* 1978, 1980; Kaczmarek and Strumwasser, 1981, 1984), but the Na^+ current in bag cells, for example, has been assumed to be similar to other *Aplysia* Na^+ currents (Adams and Gage, 1979) and has not been described. Ca^{2+} currents have been described in terms of their single-channel conductance (Strong *et al.* 1987) but, while the sensitivity of the current to specific pharmacological agents has been reported before and after phorbol ester treatment (McCleskey *et al.* 1987; Strong *et al.* 1987), this pharmacology has been described in only one study (Nerbonne and Gurney, 1987). These reports on bag cell Ca^{2+} current have suggested that it might be similar to the L-type Ca^{2+} currents. Since the basic bag cell Ca^{2+} current is modified by protein kinase C, resulting in the appearance of a new Ca^{2+} channel type (Strong *et al.* 1987), a more complete characterization of both Ca^{2+} currents is warranted.

The bag cells provide a model for the study of peptide modulation of both excitability and repetitive firing, but an understanding of modulation depends on a full characterization of the inward currents.

Materials and methods

Aplysia were obtained from the University of Miami Aplysia Facility, where they were raised from eggs laid by wild-caught brood stock. *A. californica* and *A. brasiliana* of known ages from community tanks maintained at 15 °C were brought into the laboratory, where they were closely observed in flow-through aquaria at 20 °C. Since the objective of this study was to characterize the ionic currents relevant to cells naturally capable of afterdischarge, all experiments were performed on cells from sexually mature animals, designated as such if they were observed mating and/or laying eggs. Experiments were carried out within 2 weeks of the first observed mating.

Experiments to assess whole-cell ionic currents were executed with the whole-cell variation of the patch-clamp technique (Hamill *et al.* 1981) on cells maintained in short-term tissue culture at a room temperature of 19–21 °C. Abdominal ganglia with intact bag cell clusters were removed from animals anesthetized for 1 h in a 1:1 mixture of isotonic $MgCl_2$ and sea water and were digested in an enzyme solution in high-salt L-15 medium for 20–24 h at room temperature. The enzyme solution consisted of 12.5 mg of neutral protease, 5 mg of hyaluronidase and 1 mg of collagenase type XI (all from Sigma, St Louis, MO, USA) in 5 ml of L-15 (Gibco, Grand Island, NY, USA) adjusted to 925 mosmol kg^{-1} by the addition of (in mmol l^{-1}) 300 NaCl, 4 KCl, 20 $MgCl_2$, 20 $MgSO_4$, 8 $CaCl_2$, pH 7.4–7.7. After digestion, the connective tissue sheath was removed from the clusters and the bag cells were dispersed onto 35 mm culture dishes (Falcon) in high-salt L-15 medium, plus 100 units 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 24–48 h later. Electrophysiological recordings were made using an Axopatch 1D, using the PClamp programs (Axon Instruments, Foster City, CA, USA) for data acquisition and storage on an 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, 0.3 GTP and 40 Hepes–CsOH, pH 7.4. When filled with this solution of approximately 930 mosmol kg^{-1}, pipette resistances were 0.5–0.8 MΩ. For recording Na^+ and K^+ currents, artificial sea water (ASW) was used (in mmol l^{-1}): 417 NaCl, 55 $MgCl_2$, 10 $CaCl_2$, 10 KCl, 10 Hepes–KOH, pH 7.6. The 0 % Na^+ current level was established by recording in (in mmol l^{-1}): 10.4 KCl, 55 $MgCl_2$, 11 $CaCl_2$, 460 Tris–KOH, pH 7.6. The Ca^{2+} current was not evident during Na^+ current recordings because of its relatively greater latency and its small amplitude in 10 mmol l^{-1} Ca^{2+}. For studying 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 0 % Ca^{2+} current level was established by recording in this solution plus 100 μmol l^{-1} Cd^{2+}. The cell was bathed in different solutions during an experiment by applying them from 1 μl pipettes attached to solution reservoirs. Control recordings, likewise, were obtained in a solution flowing from a 1 μl pipette. All voltage-activated currents were elicited from a holding voltage of −70 mV or from a more depolarized voltage, by test depolarizations with a duration of 50 ms to 1 s separated by a pause of 4–20 s. Compensations for whole-cell capacitance and series resistance were always made at the amplifier. Following approximately 80 % series resistance compensation, series

resistance values were less than 2 MΩ. The Na^+ and Ba^{2+} currents studied were less than 8 nA, and voltage errors were less than 3 mV. These errors were not corrected in the data presented. The Na^+ and Ba^{2+} currents were often studied in the same cells, and the activation range for Ba^{2+} currents was consistent with high-threshold Ca^{2+} currents, so it was believed that the activation range for Na^+ currents was a true feature of these cells and not a result of a poor voltage-clamp. The PClamp 5 P/4 leak subtraction protocol was used in some of the data presented.

All reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise noted. Phorbol 12-myristate 13-acetate (PMA) was diluted into ASW from a 1 μmol l^{-1} stock solution in 20% dimethyl sulphoxide (DMSO) kept frozen until use. The final concentration of DMSO used was 0.5%, which was without effect on bag cell ionic currents during bath application in 1 h control experiments (N=5). Tetrodotoxin (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 appropriate extracellular solutions at the time of experiments. Nifedipine and ω-conotoxin were obtained from Calbiochem, San Diego, CA, USA. Nifedipine was diluted daily from stock solutions of 5 mmol l^{-1} in ethanol made bimonthly and stored at −4 °C in foil-wrapped vials, and was kept in the dark. Solutions of ω-conotoxin were made up daily. The ω-conotoxin was applied to cells in a solution containing low levels of divalent ions consisting of L-15 to which 397 mmol l^{-1} NaCl had been added. Each drug was bath-applied from a 1 μl pipette during a series of depolarizing episodes separated by 8 s intervals at the holding potential, after control recordings had been collected. After studying the effects of the drug, washout of its effects using extracellular solution was attempted.

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.

Results

In addition to the K^+ currents described (Kaczmarek and Strumwasser, 1984), 90% of bag cells from sexually mature animals also had inward currents for Na^+ or Ca^{2+} or both. Na^+ currents were recorded in 73% of mature cells and Ca^{2+} currents in 87%. Outward currents, evoked from a holding potential of −70 mV, displayed cell-to-cell variability in the proportion of fast-activating to inactivating current observed. This may have been due to variations in the relative amounts of A-type K^+ current, delayed rectifier K^+ current and Ca^{2+}-activated K^+ current, which have been described in bag cells by Kaczmarek and Strumwasser (1984).

Action potentials could be elicited in bag cells by current injection before rupture of the membrane patch; after establishing the whole-cell recording configuration, it was possible to measure either Na^+ current or both Na^+ and Ca^{2+} currents. There appeared to be no differences between *A. californica* and *A. brasiliana* inward currents, so data from both species are presented in the results that follow, with the species designated accordingly.

Na$^+$ *currents*

The current recordings in Fig. 1A from an *A. californica* bag cell show that Na^+ currents activated at approximately −30 mV and peaked within 2 ms during a depolarizing test pulse. Na^+ current amplitude peaked somewhere between +10 and +20 mV as shown in the current–voltage relationship (*I–V*) of Fig. 1B. The average zero current potential was close to the Na^+ equilibrium potential (E_{Na^+}) of +84 mV. The latency, duration and voltage activation range of bag cell Na^+ currents

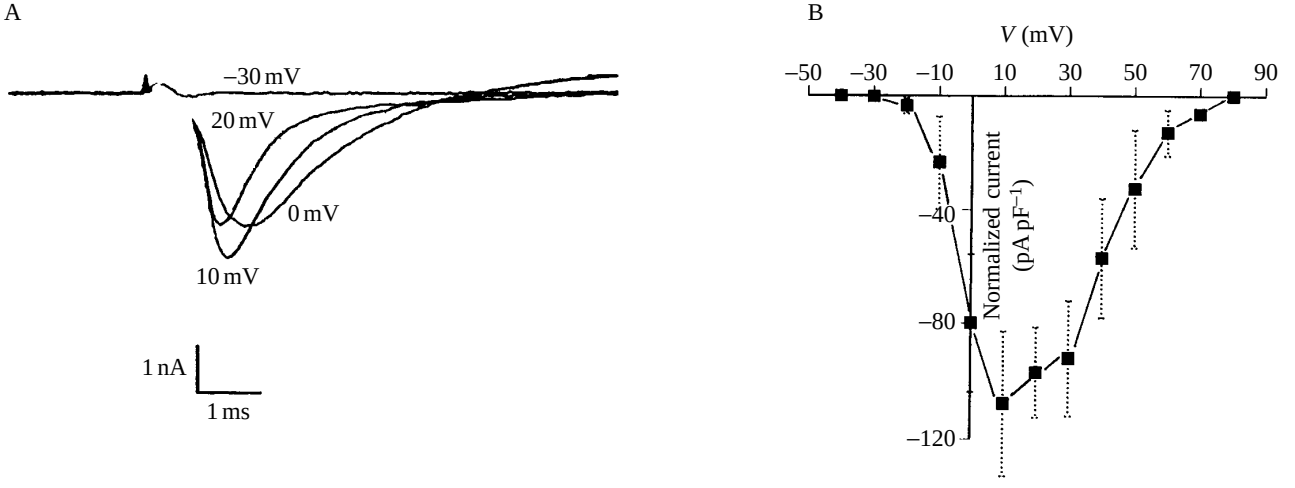

Fig. 1. Na^+ currents in cultured bag cells from sexually mature *Aplysia californica*. Whole-cell voltage-clamp recordings in ASW. (A) Na^+ currents at the indicated test potentials. Holding potential −70 mV. Test potentials lasted for 50 ms and occurred at 4 s intervals. Approximately 0.5 ms segments of the capacitative transient of certain recordings are not visible. (B) Average current–voltage (*I–V*) relationship for Na^+ current expressed as a function of whole cell capacitance in 12 bag cells (means ± S.D.).

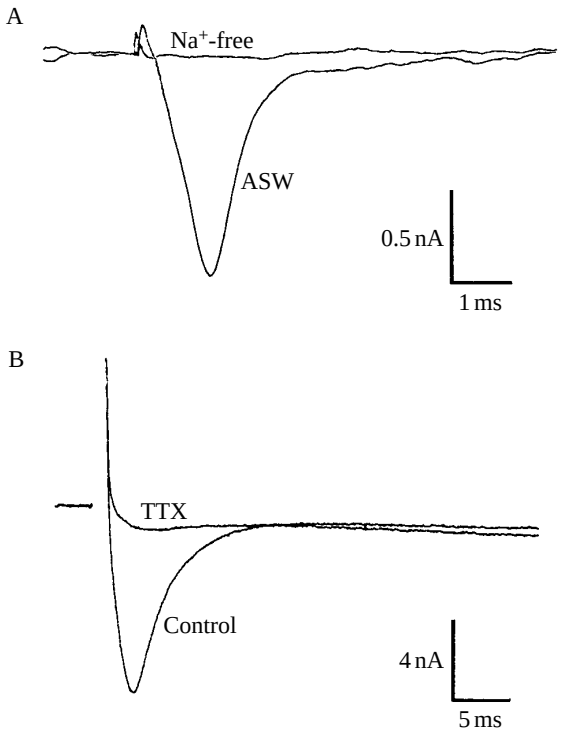

Fig. 2. Na^+ currents were dependent on extracellular Na^+ and blocked by tetrodotoxin (TTX). (A) Na^+ currents at +10 mV in ASW and upon exposure to a Na^+-free extracellular solution from a 1 μl pipette. (B) Na^+ currents at +10 mV in ASW and upon exposure to 30 nmol l^{-1} TTX in ASW from a 1 μl pipette.

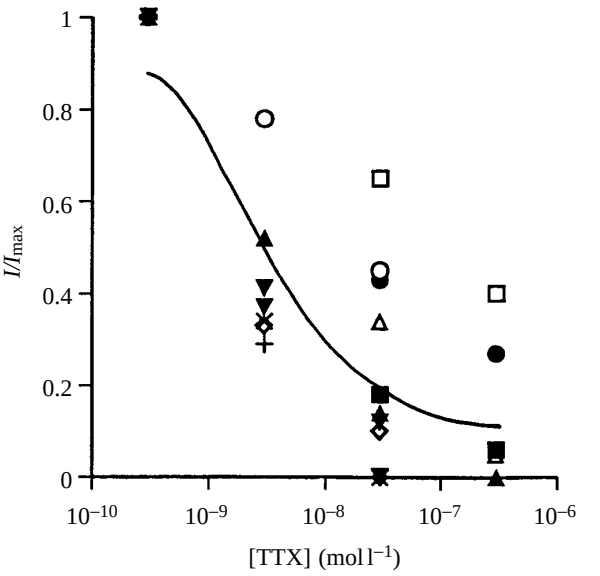

Fig. 3. Dose–response relationship for the inhibition of bag cell Na^+ current by tetrodotoxin (TTX). Data were collected in 13 cells, designated by different symbols, but not all concentrations were tested in every cell. The curve was fitted by the equation $I/I_{max}=[1-(nB/K_D)]/[1+(B/K_D)]$, where I is the current amplitude, B is the concentration of TTX, n is the Hill coefficient and K_D is the half-inhibitory concentration. K_D=2.6 nmol l^{-1}, n=−0.9.

were similar in *A. californica* and *A. brasiliana* and were also similar to those of Na^+ currents measured in neurone R_{15} of *A. juliana* (Adams and Gage, 1979).

The Na^+ currents recorded in ASW were abolished when the bathing solution was exchanged for one lacking Na^+ (Fig. 2A) and were restored upon return to ASW (not shown). In addition, the currents were blocked by tetrodotoxin (TTX), a Na^+-channel-specific antagonist (Fig. 2B). The dose–response relationship for the block of the Na^+ current by TTX is shown in Fig. 3. Bag cell Na^+ current showed a sensitivity to block by TTX similar to that of mammalian neurones, with a K_D of 2.6 nmol l^{-1}. Block by TTX was reversible. The Na^+ current was unaffected by external Cd^{2+} (100 μmol l^{-1}; two cells) and nifedipine (5 μmol l^{-1}; one cell), two agents that inhibited Ca^{2+} current in these cells (data not shown).

The sensitivity of bag cell whole-cell Na^+ current to a depolarized holding potential (V_h) and its recovery from steady-state inactivation are features useful both for comparisons with mammalian currents and for estimating the probable contribution of a current to depolarizations causing both action potentials and neurosecretion. Sensitivity to V_h was evaluated in two ways. Families of currents were recorded at depolarized test potentials from different V_h (Fig. 4A) and currents were recorded at a single test potential from different V_h maintained for 1 s prior to the test pulse (Fig. 4B). Fig. 4A from an *A. californica* bag cell shows that current amplitude is approximately 65 % of peak amplitude at a V_h of −30 mV. A plot of the Na^+ current amplitude as a function of V_h maintained for 1 s is shown in Fig. 4B. At a test potential of +10 mV, the calculated half-inactivation voltage ($V_{1/2}$) was −21 mV. Similar values for $V_{1/2}$ were obtained for other voltages near the activation peak. This contrasts with most fast, transient Na^+ currents, which half-inactivate under a similar protocol at approximately −65 mV, but resembles both neurone R_{15} (Adams and Gage, 1979) and neurones in the developing mammalian CNS (Hoehn *et al.* 1993). Fig. 4C shows recovery from inactivation using a double pulse protocol. Time constants were 2.9 ms at V_h=−70 mV and 6.8 ms at V_h=−30 mV.

Ca^{2+} currents

Studies on vertebrate Ca^{2+} channels have resulted in the recognition of at least four types of Ca^{2+} current on the basis of their activation range, pharmacology, kinetics and single-channel conductance, and these have been designated T-, L-, N- and P-types (Bean, 1989; Llinás *et al.* 1989). Results from several pharmacological investigations and measurements of single-channel conductances suggest that it may be possible to characterize bag cell Ca^{2+} channels according to these known vertebrate types. A description of the current in kinetic and pharmacological terms is important for understanding the effects of protein kinase C enhancement of the Ca^{2+} current, which is believed to recruit a new channel type (Strong *et al.* 1987). Since even vertebrate Ca^{2+} currents do not always fit

the appropriate stereotypic profiles (Fox *et al.* 1987) in every respect, it is important to extend the pharmacological reports on bag cell Ca^{2+} currents to include measurements of their time course and sensitivity to V_h.

Data on Ca^{2+} currents in sexually mature bag cells are shown in Figs 5, 6 and 7. Ca^{2+} currents activated at test potentials less negative than approximately −10 mV and peaked at approximately +20 mV when 11 mmol l^{-1} Ba^{2+} was used as the charge carrier (Fig. 5A,B, showing examples in *A. californica*). The high voltage activation threshold of bag cell Ca^{2+} current (Fig. 5B) and its block by Cd^{2+} (100 μmol l^{-1}), but not by Ni^{2+} (100 μmol l^{-1}), suggest that it may be similar to L-, N- or P-type currents. In these characteristics and in those that follow, *A. californica* and *A. brasiliana* did not differ.

Among the high-threshold channel types in vertebrates, the L-type Ca^{2+} current is often sustained during a long depolarization (Bean, 1989). Bag cell whole-cell Ca^{2+} current was often maintained during a 200 ms depolarizing test pulse (Fig. 5A). 49 % of bag cell Ca^{2+} currents maintained at least 50 % of peak current at the end of the pulse. In some cells, it was difficult to block all outward currents active at the test voltage, despite the inclusion of K^+ channel blockers in the extracellular solution, and this may have contributed to the decline of maximum inward current amplitude during a test pulse in some cells.

Sensitivity to dihydropyridines (DHPs) distinguishes vertebrate L-type from N- and P-type Ca^{2+} currents (Bean, 1989; Llinás *et al.* 1989). Nerbonne and Gurney (1987) illustrated that the DHP blocker nifedipine (10 μmol l^{-1}) blocked the bag cell Ca^{2+} current in two cells by an average of more than 90 %. Strong *et al.* (1987) reported that Ca^{2+} currents, with and without phorbol ester treatment, were blocked equally by nifedipine, but the percentage block was not reported. The effects of nifedipine (10 μmol l^{-1}) on the bag cell Ca^{2+} current was tested in 25 cells; it was found irreversibly to inhibit a

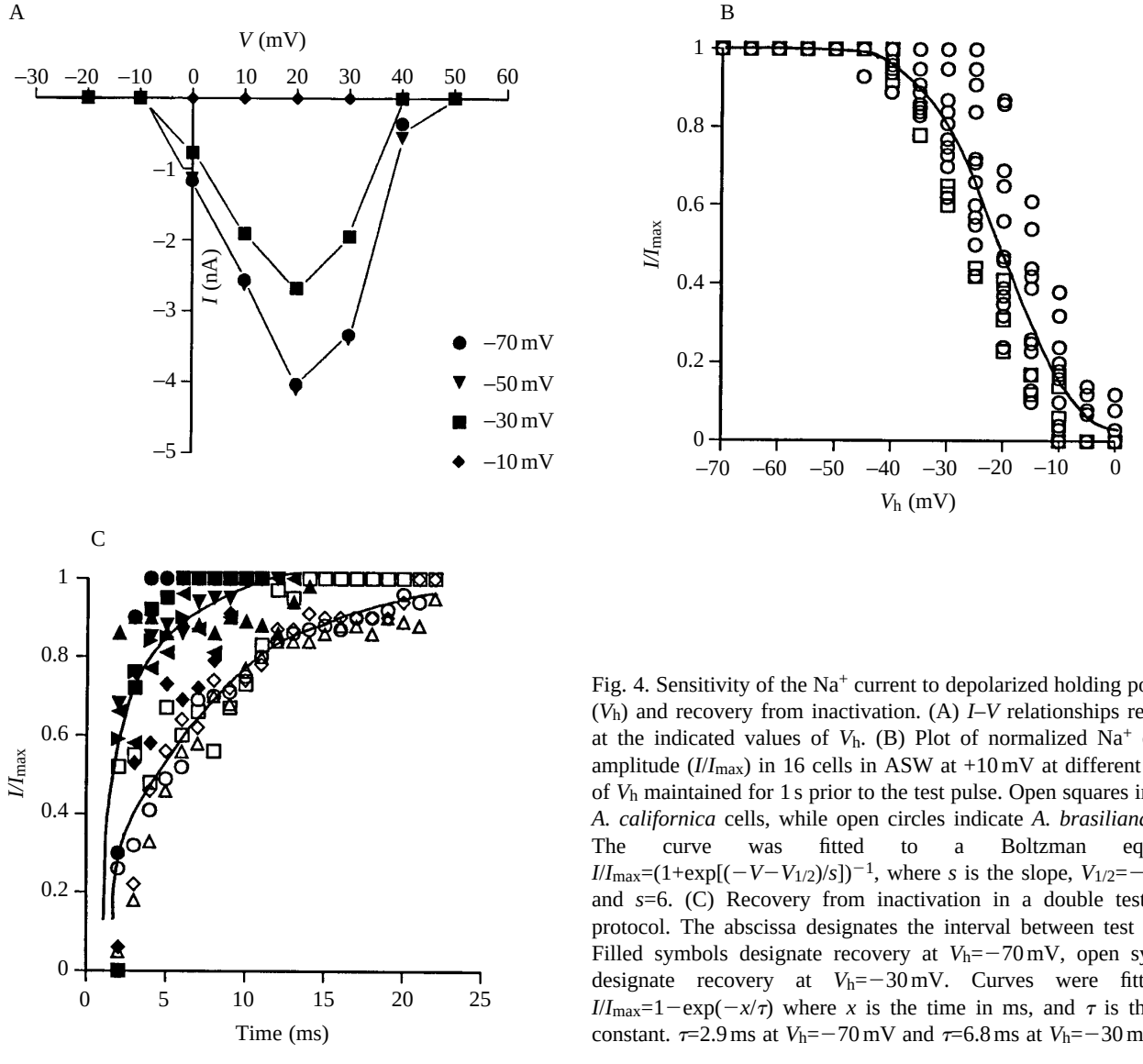

Fig. 4. Sensitivity of the Na^+ current to depolarized holding potential (V_h) and recovery from inactivation. (A) *I–V* relationships recorded at the indicated values of V_h. (B) Plot of normalized Na^+ current amplitude (I/I_{max}) in 16 cells in ASW at +10 mV at different values of V_h maintained for 1 s prior to the test pulse. Open squares indicate *A. californica* cells, while open circles indicate *A. brasiliana* cells. The curve was fitted to a Boltzman equation, $I/I_{max}=(1+\exp[(-V-V_{1/2})/s])^{-1}$, where s is the slope, $V_{1/2}=-21$ mV and $s=6$. (C) Recovery from inactivation in a double test pulse protocol. The abscissa designates the interval between test pulses. Filled symbols designate recovery at $V_h=-70$ mV, open symbols designate recovery at $V_h=-30$ 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. $\tau=2.9$ ms at $V_h=-70$ mV and $\tau=6.8$ ms at $V_h=-30$ mV.

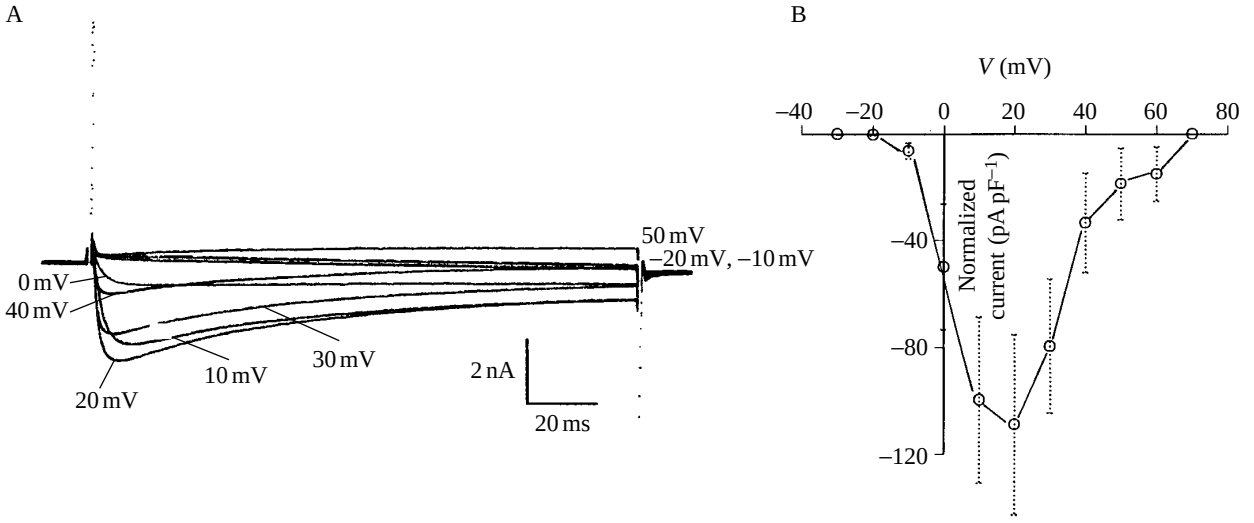

Fig. 5. Ca^{2+} currents in *Aplysia californica* bag cells from mature animals. (A) Ca^{2+} currents recorded in Ba^{2+}–Cs^{+}–TEA^{+} at the test potentials indicated. Holding potential −70 mV. Test potentials lasted for 200 ms and occurred at 6 s intervals. (B) Current–voltage (*I–V*) relationship for Ca^{2+} current expressed as a function of whole-cell capacitance in 12 bag cells (means ± S.D.).

variable percentage of the current. This variability of block is illustrated in Fig. 6A,B. The average inhibition by nifedipine in *A. californica* was 42±32 % (mean ± S.D.; *N*=21) and in *A. brasiliana* was 42±39 % (*N*=4). Bag cell Ca^{2+} current was not, however, inhibited by another DHP, nimodipine (5 μmol l^{-1}; *A. californica*, *N*=2 cells) or enhanced by the DHP agonist Bay K 8644 (5 μmol l^{-1}; *A. californica*, *N*=3 cells, and *A. brasiliana*, *N*=3 cells) (data not shown). The lack of effect of Bay K 8644 on *Aplysia* bag cell Ca^{2+} currents was also observed in *A. californica* by Nerbonne and Gurney (1987).

Vertebrate N-type Ca^{2+} current is blocked by the marine toxin ω-conotoxin, while L- and P-type currents are unaffected (Bean, 1989; Llinás *et al.* 1989). McCleskey *et al.* (1987) reported that the bag cell Ca^{2+} current was insensitive to ω-conotoxin. The effects of 10 μmol l^{-1} bath-applied ω-conotoxin were tested in 12 bag cells in the present experiments. In nine cells (six cells in *A. californica* and three cells in *A. brasiliana*), ω-conotoxin irreversibly inhibited approximately 10 % of whole-cell Ca^{2+} current (Fig. 6C, which shows an *A. brasiliana* cell); in two cells, the inhibition was complete, while in one cell ω-conotoxin had no effect (the latter three cells were all from *A. brasiliana*). The majority of these data support the report described.

An additional distinguishing feature of the L-type Ca^{2+} current from the N-type Ca^{2+} current in many cell types is its relative insensitivity to depolarized V_h. Since the results of the pharmacological experiments were ambiguous, the effects of V_h on the whole-cell current were examined. The *I–V* relationships in Fig. 7A in a cell from *A. californica* recorded at different values of maintained V_h show that almost all of the peak current at V_h of −70 mV is available at a V_h as depolarized as −40 mV. The same was true of Ca^{2+} currents in *A. brasiliana*. The plot of V_h maintained for 1 s before the test pulse *versus* whole-cell current amplitude is shown in

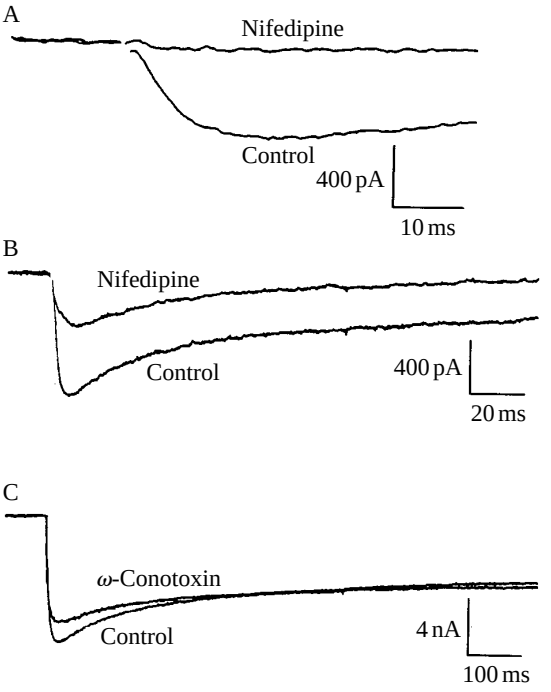

Fig. 6. Inhibition of Ca^{2+} currents by nifedipine and ω-conotoxin. (A) Ca^{2+} currents recorded at +20 mV in Ba^{2+}–Cs^{+}–TEA^{+} and in 5 μmol l^{-1} nifedipine in Ba^{2+}–Cs^{+}–TEA^{+}. This degree of inhibition was observed in a minority of bag cells. (B) Ca^{2+} currents recorded in another cell at +20 mV in Ba^{2+}–Cs^{+}–TEA^{+} and after exposure to 10 μmol l^{-1} nifedipine. This degree of inhibition by nifedipine was observed in the majority of bag cells. (C) Ca^{2+} currents recorded in another cell at +20 mV in Ba^{2+}–Cs^{+}–TEA^{+} and after exposure to 10 μmol l^{-1} ω-conotoxin in L-15 solution containing a low concentration of divalent cations. The cell was returned to Ba^{2+}–Cs^{+}–TEA^{+} before recording the second trace. This degree of inhibition was observed in nine of the 12 bag cells exposed to ω-conotoxin.

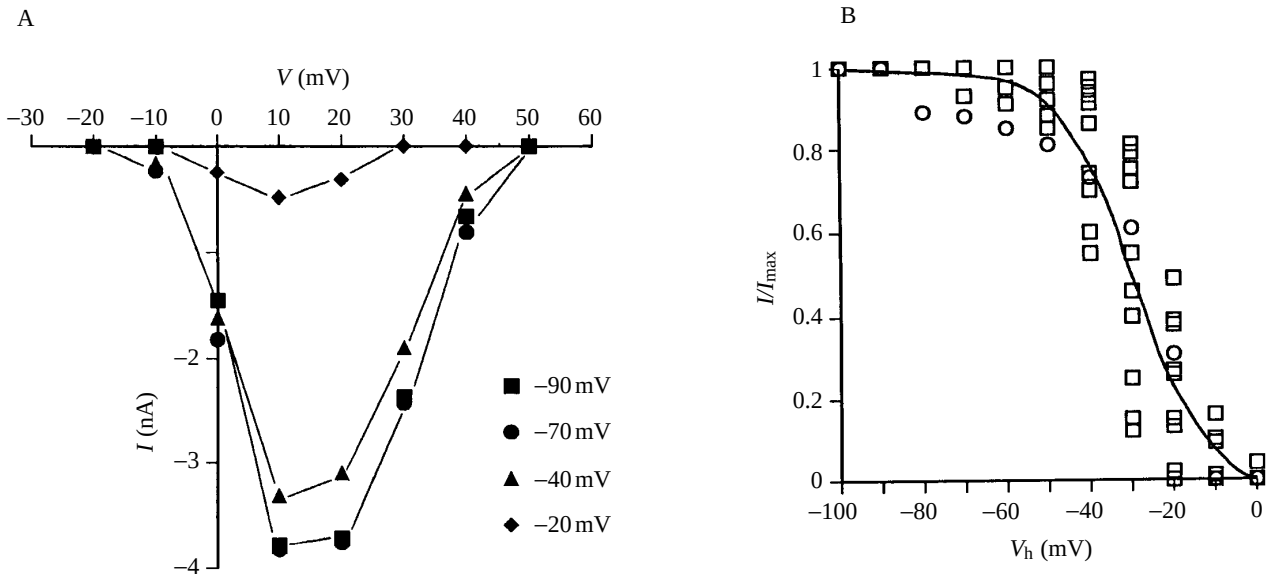

Fig. 7. Sensitivity of the Ca^{2+} current to depolarized V_h. (A) *I–V* relationships recorded at the indicated V_h. (B) Plot of normalized Ca^{2+} current amplitude (I/I_{max}) at +20 mV in Ba^{2+}–Cs^{+}–TEA^{+} in 11 cells at different V_h maintained for 1 s prior to the test pulse. Open squares indicate *A. californica* cells while open circles indicate one *A. brasiliana* cell. The curve was fitted to a Boltzman equation with $V_{1/2}$=−30 mV, *s*=7.8 (see Fig. 4).

Fig. 7B. These data are fitted by a single Boltzman distribution, suggesting a uniform population of channels, with a $V_{1/2}$ of −30 mV.

The profile of an average bag cell Ca^{2+} current in these kinetic and pharmacological terms (its time course, activation range, insensitivity to depolarized V_h and relative susceptibility to block by nifedipine but not by ω-conotoxin) suggests that most of the current is carried by channels that are more similar to the L-type than to other types of vertebrate Ca^{2+} currents. This classification is approximate and it serves merely to establish a baseline against which the modulated Ca^{2+} current can be compared.

The insensitivity of the Ca^{2+} current to depolarized holding voltages indicates that, like the Na^{+} channels, most bag cell Ca^{2+} channels would be available to open upon depolarization of the cell membrane from a resting potential as depolarized as −30 mV. The data presented so far indicate that bag cells have the voltage-gated channels required to cause depolarization during an action potential in afterdischarge.

Modulation of voltage-gated currents

Increases in bag cell Ca^{2+} currents due to activation of protein kinase C have been studied at the single-channel and whole-cell level in matched sets of control and phorbol-ester-treated cell cultures (DeRiemer *et al.* 1985*b*; Strong *et al.* 1987). This form of modulation of the Ca^{2+} current was confirmed by recording from cells from an individual *A. brasiliana*. Maximum Ca^{2+} current amplitude from cells in control cultures ranged from 0.67 to 4.6 nA, while that in cells from cultures treated for 1 h with 25 nmol l^{-1} PMA ranged from 2.0 to 9.7 nA.

It has been demonstrated that the action potentials of an individual cell are enhanced by phorbol esters (DeRiemer *et al.* 1985*b*; Conn *et al.* 1989*a*,*b*), but increases in the whole-cell Ca^{2+} currents in individual cells before and after treatment with phorbol ester have not been confirmed. The results of two experiments on whole-cell currents in individual bag cells are illustrated in Fig. 8. These cells were representative of a total of five such experiments in which bag cell currents were recorded in individual cells before and after phorbol ester treatment.

Fig. 8A shows whole-cell ionic currents in a bag cell from a mature *A. brasiliana* in ASW (top sets of traces) and in Ba^{2+}–Cs^{+}–TEA^{+} (bottom sets of traces). Only outward currents were macroscopically visible in this cell. After recording the currents in Fig. 8A, the dish containing this cell was bathed for 1 h in 25 nmol l^{-1} PMA and then washed with fresh ASW. The currents in Fig. 8B were then recorded using a new recording pipette. PMA treatment for 1 h increased the outward currents by approximately 60 % and revealed currents for Na^{+} and Ca^{2+} that were not visible in the control recordings. These effects were not observed after 30 min in PMA. In five control experiments in which DMSO, the agent used to dissolve PMA, was bath-applied for 1 h after control recordings had been collected, current amplitudes were unaffected (⩽±10 %) when the cell was subsequently voltage-clamped using a new recording pipette. Whole-cell *I–V* relationships in Fig. 8C,D, from another experiment on an *A. californica* bag cell, show that PMA increases macroscopic Na^{+} and Ca^{2+} currents at all test potentials in the activation range, without marked shifts in the activation range. In each of the five cells tested, PMA increased the inward currents by at least 52 %. Passive leak during the period of PMA exposure did not exceed ±12 %. These data confirm the phorbol-ester

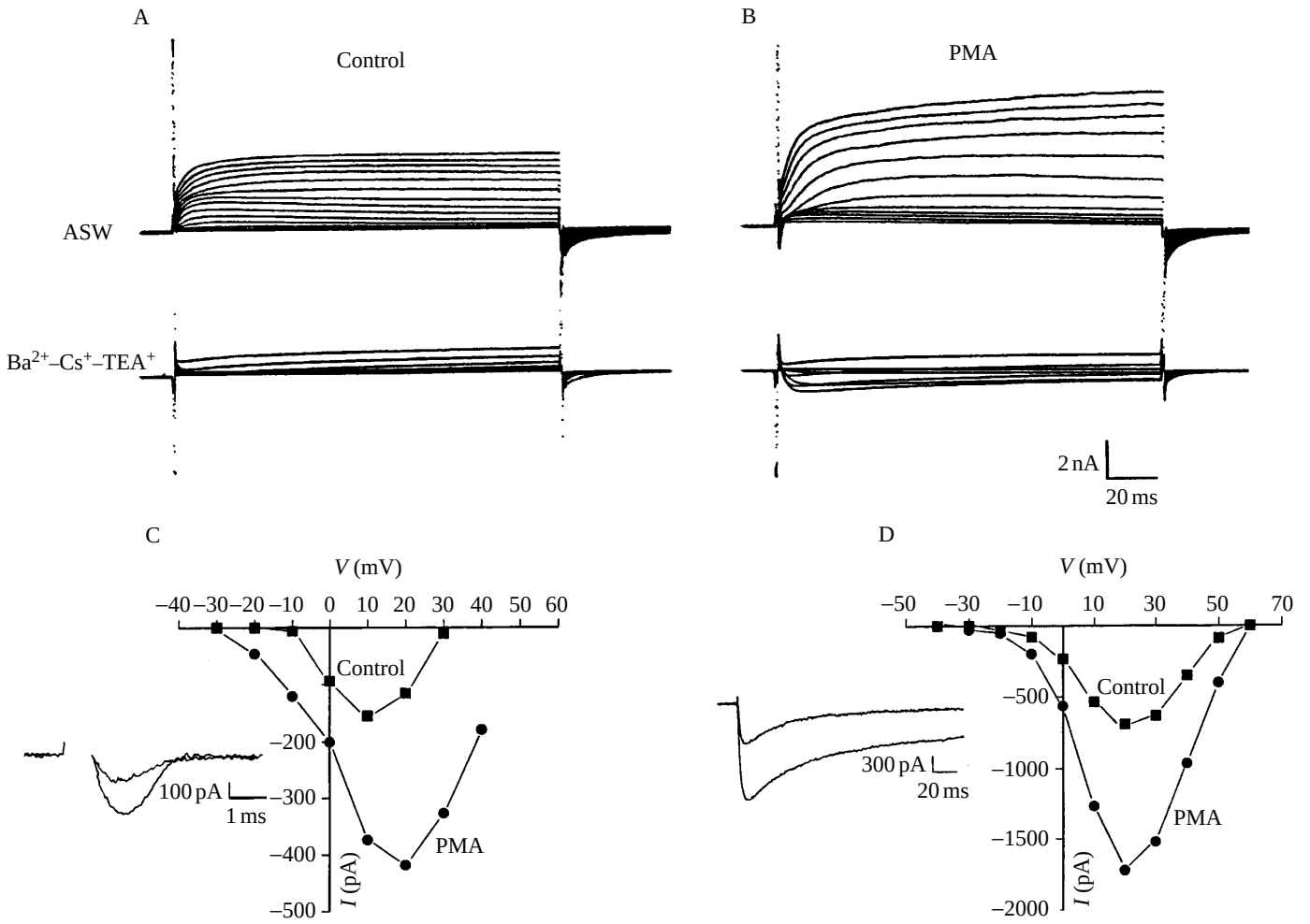

Fig. 8. Ionic currents in two *Aplysia* bag cells before and after exposure for 1 h to a membrane-permeant activator of protein kinase C. The top sets of traces in A and B were recorded in ASW at potentials from −70 to +70 mV (A) and from −70 to +40 mV (B). The bottom sets of traces in A and B were recorded in Ba^{2+}–Cs^{+}–TEA^{+} at potentials from −30 to +40 mV. The scale is identical for all sets of traces in A and B. (A) Currents before treatment with phorbol 12-myristate 13-acetate (PMA). (B) Currents recorded in the same cell after 1 h in 25 nmol l^{-1} PMA. The amplitude of outward currents is increased compared with control recordings, and inward current is now visible in both ASW (Na^{+} current) and in Ba^{2+}–Cs^{+}–TEA^{+} (Ca^{2+} current). The data in C and D were collected in another cell before and after 1 h of PMA treatment. V_h=−70 mV. (C) Na^{+} current *I–V* relationships before and after PMA treatment. Inset shows currents recorded at +10 mV before (upper recording) and after PMA treatment. Segments of the capacitative transient of the recordings shorter than 0.5 ms are not visible. (D) Ca^{2+} current *I–V* relationship before and after PMA treatment. Inset shows currents recorded at +20 mV before (upper recording) and after PMA treatment.

enhancement of Ca^{2+} current seen by other investigators and demonstrate that Na^{+} current and outward current can both be enhanced by phorbol esters.

Examples of experiments to characterize phorbol-ester-modulated Na^{+} and Ca^{2+} currents in *A. californica* are shown in Fig. 9. *I–V* relationships recorded at different V_h values after PMA treatment show that there was no change in the sensitivity of Na^{+} (Fig. 9A) and Ca^{2+} currents (Fig. 9B) to depolarized V_h after 1 h of PMA treatment. This was also apparent in Boltzman fits of the plots of the Na^{+} and Ca^{2+} current amplitudes after PMA treatment as a function of V_h maintained for 1 s in four and six cells, respectively (data not shown). The $V_{1/2}$ for Na^{+} current was −23 mV (slope=5), and for Ca^{2+} current was −27 mV (slope=8). The Boltzman functions fitted to these data were similar to those obtained in the control experiments shown in Figs 4 and 7, without PMA treatment.

Fig. 9C,D shows the effects of nifedipine and ω-conotoxin, respectively, on Ca^{2+} current in PMA-treated cells. Inhibition by nifedipine (10 μmol l^{-1}) was approximately 50 % (N=5), while ω-conotoxin (10 μmol l^{-1}) had no effect on the current amplitude (N=4). The similarity in the pharmacology of control and PMA-induced Ca^{2+} currents as well as their lack of sensitivity to V_h suggest that PMA enhances the same currents for Na^{+} and Ca^{2+} present in control recordings.

Discussion

The bag cell afterdischarge begins with a depolarization of

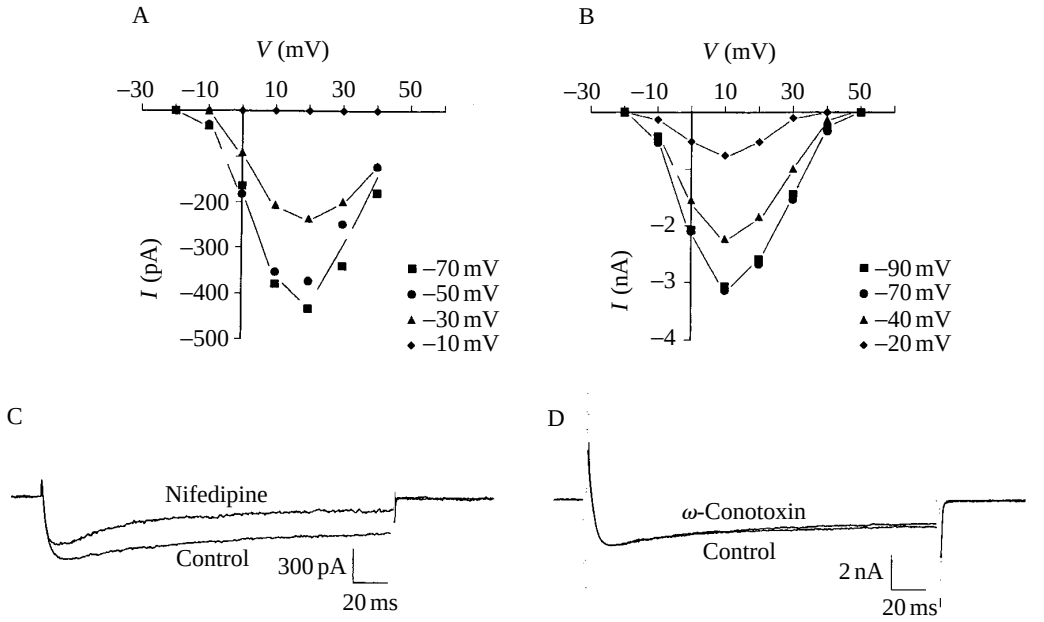

Fig. 9. Characteristics of Na^+ and Ca^{2+} currents in *Aplysia* bag cells exposed to phorbol ester. (A) Na^+ current *I–V* relationships recorded at the indicated V_h. (B) Ca^{2+} current *I–V* relationships recorded at the indicated V_h. (C) Ca^{2+} currents recorded at +20 mV in Ba^{2+}–Cs^+–TEA^+ and after exposure to 10 μmol l^{-1} nifedipine. This degree of inhibition was typical of five cells. (D) Ca^{2+} currents recorded in another cell at +20 mV in Ba^{2+}–Cs^+–TEA^+ and after exposure to 10 μmol l^{-1} ω-conotoxin in L-15 solution containing a low concentration of divalent cations. The cell was returned to Ba^{2+}–Cs^+–TEA^+ before recording the second trace.

the cells from a resting potential of approximately −60 mV by an, as yet, unknown stimulus to a threshold level for the first action potential. It has been reported that, very early during in the afterdischarge, the resting membrane potential of bag cells depolarizes to a new value of approximately −30 mV (Fisher and Kaczmarek, 1990). The activation range, time of recovery from inactivation and the $V_{1/2}$ of the Na^+ current measured in cultured bag cells could account for this current carrying the upstroke of each spontaneously produced action potential during the Na^+-dependent phase of afterdischarge, when firing is as rapid as 2–4 Hz (Dudek and Kossatz, 1982), if the resting potential was −30 mV. For membrane potentials more negative than −30 mV, it is possible either that an additional inward current, active near the resting potential, depolarizes the cell to the activation threshold for Na^+ current (Wilson and Kaczmarek, 1993) or that the activation range for Na^+ current shifts to a more hyperpolarized potential; for example, in response to a change in the level of an intracellular second messenger. The activation range of Na^+ channels has been shown to be modified by G-protein activation (Schubert *et al.* 1989) and, specifically, by protein kinase C activation (Numann *et al.* 1991; Dascal and Lotan, 1991). Protein kinase C is a known modulator of ionic currents in bag cells during afterdischarge, but PMA was not observed to cause a shift in the activation range of whole-cell Na^+ or Ca^{2+} currents in cultured cells.

The high sensitivity of the bag cell Na^+ current to TTX, with a K_D of 2.6 nmol l^{-1}, was somewhat unexpected given the micromolar concentrations often used to achieve complete block of Na^+ current in invertebrate preparations (see, for example, Adams and Gage, 1979; Geduldig and Junge, 1968). However, Narahashi *et al.* (1964) found that 15 nmol l^{-1} TTX blocked the Na^+ conductance in lobster giant axons, so precedents for a high sensitivity of invertebrate preparations to TTX have been found.

The pharmacological results on the Ca^{2+} currents, as well as their lack of sensitivity to depolarized V_h, suggest that the channels with or without modulation by phorbol ester are similar. These channels bear more resemblance to vertebrate L-type channels than to other vertebrate types, but do not closely fit the criteria for L-type channels. It is possible that the lack of complete inhibition of Ca^{2+} currents by nifedipine in most cells is due to the presence of another Ca^{2+} channel type, but this is not supported by single-channel studies (Strong *et al.* 1987). Another possibility is a lower dihydropyridine sensitivity of these channels in invertebrate cells, resulting, for example, in a less complete shift to a gating mode with a very low probability of channel opening in the presence of DHP blockers (Hess *et al.* 1984). This possibility is supported by the lack of effect of Bay K 8644 on bag cells.

The observed phorbol-ester-induced enhancements of individual bag cell Ca^{2+} current amplitudes were expected, since they are consistent with reports comparing matched control and PMA-enhanced Ca^{2+} currents (DeRiemer *et al.* 1985*b*; Strong *et al.* 1987). The concomitant enhancement of Na^+ current amplitude by phorbol ester is not surprising, considering that phorbol ester treatment enhanced action potential height (DeRiemer *et al.* 1985*b*). The contribution made by the Na^+ current to action potentials occurring in the second phase of the afterdischarge, at the time when

phosphoinositide hydrolysis is presumed to occur (DeRiemer *et al.* 1985*a*) is, however, uncertain. The data suggest that activation of protein kinase C does not cause the independence from Na^+ of the second phase of the afterdischarge by markedly shifting the Na^+ current activation or by increasing its sensitivity to depolarized V_h.

The data presented on the PMA-enhanced whole-cell Ca^{2+} and Na^+ currents suggest that these are similar to the currents in the absence of phorbol ester. It is possible that differences might be discernible at the single-channel level. The results with individual cells suggest that it may be possible to observe single-channel currents in a membrane patch and the subsequent changes after protein kinase C activation.

A detailed study of the modulation of outward currents was not undertaken in this study, since it has been previously addressed (Kaczmarek *et al.* 1978, 1980; Kaczmarek and Strumwasser, 1981, 1984). Enhancement of outward current by phorbol ester was not found in matched sets of control and phorbol-ester-treated cell cultures by DeRiemer *et al.* (1985*b*), so the increase observed in individual cells in this study was surprising. Whether the phorbol-ester-induced increase in outward current was due to a direct effect of phorbol ester on the current amplitude or was an indirect effect *via* enhanced activation of Ca^{2+}-induced K^+ current, which makes a substantial contribution to outward current in bag cells (Kaczmarek and Strumwasser, 1984), remains to be determined.

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