J. exp. Biol. ***185****, 267–286 (1993)*
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PROPERTIES OF VOLTAGE-ACTIVATED IONIC CURRENTS IN CELLS FROM THE BRAINS OF THE TRICLAD FLATWORM *BDELLOURA CANDIDA*

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Accepted 28 July 1993

Summary

Cells were dispersed from the brains of the triclad flatworm *Bdelloura candida* and maintained in primary culture for up to 2 weeks. Cultured cells assumed a variety of morphologies consistent with those of neurones *in vivo*. Whole-cell voltage-clamp recordings from cultured cells revealed that these cells possess a variety of ionic currents, including a fast transient sodium current, a calcium current and several potassium currents. The sodium current does not inactivate completely but instead decays to a steady-state component which has the same physiology and pharmacology as the fast transient component, suggesting that the two components are carried by the same population of channels. The physiology and pharmacology of these various currents were not remarkable save for the fact that, contrary to earlier reports, all sodium currents examined were sensitive to tetrodotoxin (TTX). These animals are, therefore, the lowest animals known to possess TTX-sensitive sodium currents and, as such, represent a major stage in sodium channel evolution.

Introduction

Platyhelminthes (the flatworms) is a pivotal phylum. In addition to forming the stem of most invertebrate lineages, its members are the simplest organisms to possess a rostral brain, and they are the lowest organisms known to display nervous system plasticity (Koopowitz and Keenan, 1982). Thus, they occupy a unique position in nervous system evolution. However, while flatworms have long been model organisms for behavioural studies (Koopowitz, 1970; Corning and Kelly, 1975; Koopowitz *et al.* 1976), we still know relatively little about the physiological properties of the cells that constitute their nervous systems. This is due, in part, to the diffuse nature of their nervous systems and also to the fact that their brains are relatively small, containing, at best, only a few

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Key words: Platyhelminthes, voltage-clamp, flatworm, tetrodotoxin, sodium current, calcium current, potassium current, *Bdelloura candida.*

hundred neurones (Bullock and Horridge, 1965). Information about the neurobiology of these animals is essential to our complete understanding of nervous system evolution and, given that these organisms lead predominantly parasitic lifestyles, to the identification of sites for new generations of anthelmintic drugs that would target this important aspect of their biology.

Intracellular recordings from neurones in the brains of several free-living species reveal that these animals are capable of producing fast, overshooting action potentials (Keenan and Koopowitz, 1981, 1984; Koopowitz, 1989). Ionic and pharmacological manipulations of these intracellularly recorded action potentials suggest that they are produced by several distinct ionic currents. These include both tetrodotoxin (TTX)-sensitive ($10 nmol\,l^{-1}$) and TTX-insensitive Na^+ channels (Keenan and Koopowitz, 1981), Cd^{2+}-sensitive Ca^{2+} channels and a Cd^{2+}-insensitive plateau potential (Keenan and Koopowitz, 1984; Solon and Koopowitz, 1982). The concurrent presence of TTX-sensitive and TTX-insensitive Na^+ channels maybe evolutionarily important, since Na^+ currents in members of the phylum Cnidaria are completely insensitive to TTX (Anderson, 1987; Meech and Mackie, 1993). To understand the properties of voltage-activated ionic currents in this important group of animals better, we have developed techniques for isolating and maintaining cells from the brains of the triclad flatworm *Bdelloura candida*. These cells were examined under voltage-clamp and found to contain a rather typical array of ionic currents, including Na^+ currents which were exclusively TTX-sensitive.

Materials and methods

Culture preparation and conditions

Bdelloura candida (Tricladia) is an ectoparasitic flatworm that lives its entire life on the legs and gills of the horseshoe crab *Limulus polyphemus*. Specimens, 0.5–1cm in length (5–8mg) were removed from the host and anaesthetized in a 1:1 solution of isotonic $MgCl_2$ and natural sea water (NSW) for 15–20min to permit washing (10s) in 10% Listerine in NSW. Anaesthesia was used only to ensure efficient and thorough disinfection of the 40 animals (see below) that were typically processed at one time, and was not used at any other stage of the dissection. After the Listerine wash, the animals were allowed to recover in NSW. The brains were then dissected free of the surrounding tissues with insect minutia pins and softened by partial digestion for 1h with collagenase (Sigma type 1A,$1.5 mg ml^{-1}$) and trypsin (Sigma type IX, $1.5 mg ml^{-1}$) in culture medium (25% L-15/75% filtered NSW, see below). The enzyme was removed with three rinses in fresh, enzyme-free culture medium. The brains were allowed to sit for another hour in culture medium and then were dissociated in 800 μl of medium by gentle trituration through a fire-polished and silanized (Sigmacote, Sigma) Pasteur pipette. A 100 μl drop containing a suspension of dispersed cells was placed on the lid of a 35mm Falcon Petri dish. The cells were allowed to settle for a period of up to 1h before being flooded with tissue culture medium. As will be shown later, the percentage of cells possessing obvious voltage-activated inward currents was very low. To compensate for this, brains from 40 animals were processed simultaneously, and the dispersed cells were

plated together in densities ranging from 150 to 200 per Petri dish lid. A typical dissociation yielded 30–40 cells per brain.

The tissue culture medium consisted of a 1:3 mixture of L-15 (Sigma) and filtered NSW. The ionic composition of the L-15 was adjusted to that of artificial sea water (ASW, see below) by adding the appropriate salts, and both it and the filtered NSW were fortified by the addition of ($mmol\,l^{-1}$): dextrose, 10; glutamine, 5; sodium acetate, 5; and Hepes buffer, 10. Each solution was adjusted to pH7.4 with NaOH. Cells were maintained in culture at 25˚C in a water-saturated air atmosphere. All tissue preparation and culture solutions contained 1% antibiotic-antimycotic (Sigma) and were filter-sterilized. Recordings could be obtained from neurones for up to 2 weeks, but the studies described here were performed on cells maintained for less than 1 week.

In an attempt to increase the proportion of cells possessing measurable inward currents (Brismar and Gilly, 1987), culture conditions were varied with respect to substratum, trophic factors and nutrients. Plastic lids and acid-washed glass coverslips were treated with a variety of artificial substrata, then evaluated for their ability to improve attachment and cell viability, as defined by cell longevity and the amplitude and type of ionic currents recorded over the life of the preparation. Untreated glass coverslips were consistently inferior to plastic. Concanavalin-A (salt free) was applied as a 5% aqueous solution for 5 min, then rinsed with distilled water and air-dried. The poly-amino acids poly-D-lysine, poly-D-glutamic acid, poly-L-serine, poly-L-ornithine, poly-L-arginine, poly-L-phenylalanine and poly-s-benzyl-L-cystine were prepared in distilled water ($0.5mg\,ml^{-1}$) and applied at $0.5\,\mu g\,cm^{-2}$. Collagen, type IV, was applied at $6\,\mu g\,cm^{-2}$. Gelatin was applied at $0.2mg\,cm^{-2}$. None of these treatments was superior to untreated plastic. The neurones attached well to concanavalin-A-treated plastic but the cells deteriorated more rapidly than those on uncoated plastic.

Several putative trophic factors were tested by adding them to the cultures after the cells had attached to plastic lids. Foetal calf serum and horseshoe crab serum (1–10%) agglutinated the cells, making them useless. Serotonin ($50nmol\,l^{-1}$) inhibited neurite outgrowth, but was otherwise without apparent effect. 7S-NGF ($20ng\,ml^{-1}$), *Hydra* head-activator peptide ($100ng\,ml^{-1}$), cyclic AMP ($1mmol\,l^{-1}$), $30mmol\,l^{-1}$ K^+, substance P ($100nmol\,l^{-1}$) and a platyhelminth peptide, neuropeptide F ($100nmol\,l^{-1}$), had no apparent effect on the viability of the cells or on the types of currents expressed (N=4 lids each). Cells survived for 2–3 days in NSW. When the volume percentage of the modified L-15 in the culture medium was increased above 25%, viability decreased; cells failed to survive overnight in 100 % L-15.

Voltage-clamp recordings

Voltage-clamp experiments were carried out using the whole-cell configuration of the patch-clamp technique (Hamill *et al.* 1981) using a Dagan 8900 patch-clamp amplifier. Pipettes were pulled from borosilicate capillary glass (Boralex, Rochester Scientific Co., Rochester, NY, USA), fire-polished and filled with one of the patch solutions given below. Offset currents were neutralized just prior to making contact with the cell membrane. Seals were obtained by gently pushing the pipette against the cell surface, applying slight suction and a $-70mV$ potential. Pipette capacitance was neutralized and

additional suction was used to rupture the cell membrane. Series resistance compensation was then added. Voltage protocols were applied and data acquisition and manipulation were carried out using an IBM AT computer equipped with a Lab Master TL-1 (100kHz) AD/DA interface and PClamp software (Axon Instruments). Capacitative and linear leakage currents were subtracted on-line, using software routines. Only cells with short processes or no processes were selected for study to ensure that the cell was adequately space-clamped. Any recordings that showed evidence of an inadequate space-clamp were discarded.

Recording solutions

Artificial sea water (ASW) had the following composition (mmol l^{-1}): NaCl, 395; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 50; Hepes, 10; pH7.4. Barium- (Ba^{2+}) and strontium- (Sr^{2+}) containing ASWs were made by direct substitution of Ca^{2+} and reduction of [Mg^{2+}] as needed. The Na^+ concentration of ASW was altered by direct substitution with choline chloride. K^+-free ASW was prepared by direct substitution with TEACl. Patch pipettes were filled with either a 'normal' intracellular saline [containing (mmol l^{-1}): KCl (or $KMeSO_3$), 440; NaCl, 30; $CaCl_2$, 1; EGTA, 11; $MgCl_2$, 3; Hepes, 20; and Pipes, 10] or a caesium (Cs^+) solution [containing (mmol l^{-1}): CsCl, 440; NaCl, 30; $CaCl_2$, 1; EGTA, 11; $MgCl_2$, 3; Hepes, 20; and Pipes, 10]. The pH of these solutions was adjusted to 7.3 with KOH or TEAOH, respectively, and the osmolality was adjusted with dextrose. All experiments were carried out at room temperature (20–22˚C).

In experiments involving changes in external saline, the clamped neurone was perfused externally from a multibarrel perfusion pipette. Tip diameters of the individual pipettes were 5–10 μm and the open end of the pipette assembly was positioned 20–30 μm from the cell under examination. Toxins and drugs were applied either by direct bath application or by perfusion pipette. The perfusate was visualized with 0.01% Fast Green, which itself exhibited no apparent effect on the currents in this study. ω-Conotoxin GVIA was purchased from RBI (Natick, MA, USA), ATX-II from Calbiochem (San Diego, CA, USA) and Neuropeptide-F was purchased from Peninsula Labs, Inc. (Belmont, CA, USA). All other toxins and drugs were supplied by Sigma Chemical Co. (St Louis, MO, USA). Numerical data are presented as mean ± S.D.; graphical data are presented as mean ± S.E.M.

Results

Morphological characteristics of isolated cells

Unipolar, bipolar and multipolar cells (Fig. 1A–C) were present in the cultures. Their processes usually appeared within 1–2h after plating and, in cultures maintained at high density for long periods, they became interwoven (Fig. 1D).

The smallest cells (5–15 μm in cell body diameter) were the most difficult to hold during recordings, and none produced any inward currents. The most abundant cells measured 15–20 μm in diameter. These were the easiest to space-clamp but only a few possessed inward currents. The largest cells (20–30 μm) were more difficult to space-clamp but they had the highest yield of inward currents. All the recordings described below were obtained from cells in the 15–30 μm size classes.

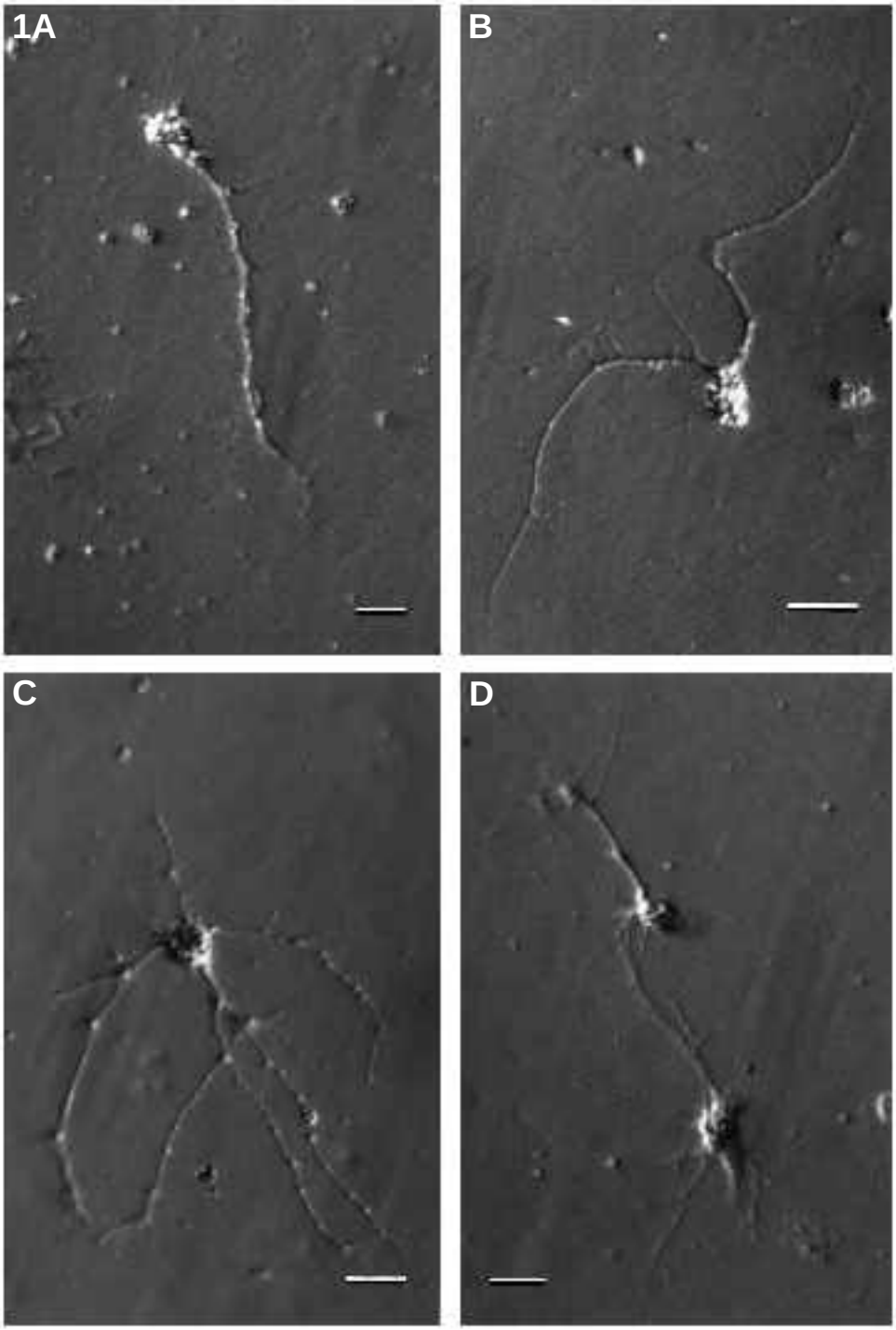

Fig. 1. Normarski micrographs of representative cells obtained from dispersed brains of *Bdelloura candida* and maintained in culture for 2h. Morphological types include unipolar (A), bipolar (B) and multipolar (C). Cells plated together frequently form apparent connections (D). Scale bars, 25μm.

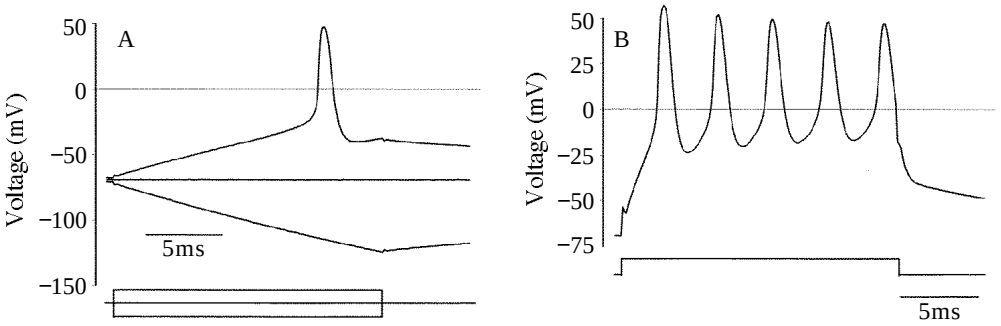

Fig. 2. Action potentials recorded from a cell from the brain of *Bdelloura candida*. The membrane potential of the cell was adjusted to −70mV by current injection. (A) Voltage responses generated by intracellular injection of 0, −200 and +200pA of current. The time course of the injected current is shown in the lower trace. (B) Repetitive action potentials evoked by injection of +1.4nA of current. Lower trace, current record (not to scale). Bathing medium, ASW; pipette solution, KCl patch solution.

Whole-cell responses

When examined using pipettes filled with KCl patch solution, cells bathed in ASW had resting membrane potentials between −45 and −70mV. Approximately 5% of the cells 15–30 μm in diameter produced action potentials when depolarized. The appearance of these action potentials was very consistent from cell to cell. All had short durations and fast rise times. The peak of these action potentials reached or exceeded +50mV (Fig. 2A). Their maximum rate of depolarization (V_{max}) was 100–120 $V s^{-1}$, and they repolarized at a maximal rate of 55–65 $V s^{-1}$. With continuous suprathreshold depolarization, action potentials occurred repetitively, at rates up to 300Hz (Fig. 2B). These action potentials were dependent on the presence of Na^+ in the bath and could be blocked by 100 μmol l^{-1} TTX (data not shown).

When these cells were examined under voltage-clamp, a greater diversity of responses became evident. Cells known to fire action potentials possessed a fast transient inward current, together with outward current (Fig. 3). A further 10–20% of the cells examined possessed a slowly activating inward current (see Ca^{2+} current section) and a complex outward current. The majority of the remaining cells exhibited complex outward currents, but no apparent inward current (see outward current section).

Inward currents

Sodium currents

All outward currents in cells bathed in 0mmol l^{-1} K^+, 400mmol l^{-1} Na^+ ASW were readily blocked with the CsCl pipette solution. Cells that produced action potentials invariably exhibited two components of inward current: a fast transient current and a non-inactivating current that ranged in amplitude from 5 to 20% of that of the fast transient current (Fig. 4A). The fast transient current activated at −16.3±5.5mV, reached maximum current amplitude at +8.6±5.3mV and reversed at +61.9±3.9mV (N=14) (Fig. 4B). The non-inactivating current exhibited similar voltage dependencies for

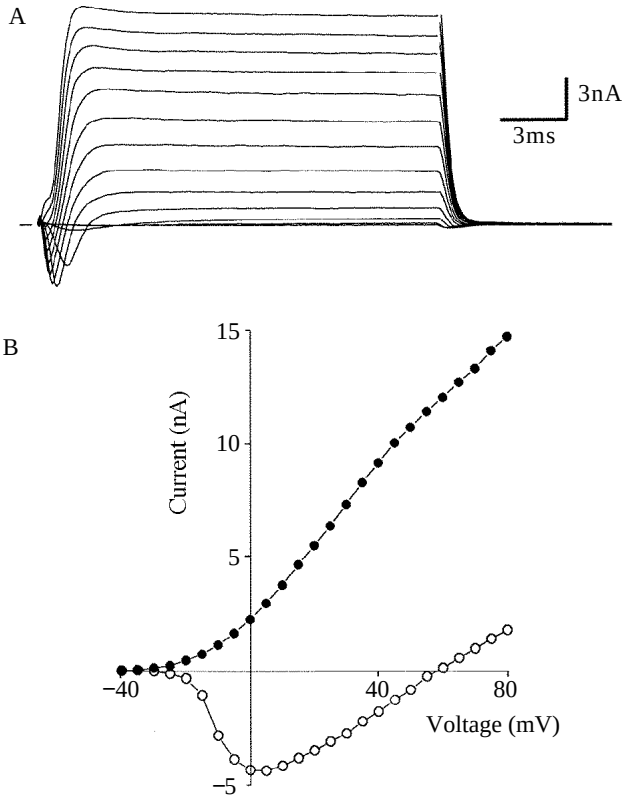

Fig. 3. Total membrane currents. (A) A cell from the brain of *Bdelloura candida* was voltage-clamped at a holding potential of −70mV. Currents were evoked by 5mV voltage steps starting at −40mV. In this and subsequent voltage-clamp records, uncorrected capacitative current artefacts at the make and break of the voltage step have been removed from the records. (B) Current–voltage plot of the peak transient inward (○) and steady-state outward (●) currents from A. Steady-state currents were measured at the end of the voltage step. Bathing medium, ASW; pipette solution, KCl patch solution.

activation and maximum current amplitude, but reversed at less positive voltages, probably as a result of Cs^+ leakage through K^+ channels. Both currents were metabolically unstable, since they diminished in size, irreversibly, with time after the whole-cell configuration had been established. Useful recordings were, therefore, limited to 20–30min. Inclusion of ATP, cyclic AMP, phorbol esters, dithiothreitol, glutathione or protease inhibitors in the pipette did not prevent this run-down.

To evaluate the ionic dependencies of these inward currents, cells expressing these two currents were voltage-clamped using pipettes filled with CsCl patch solution and then perfused extracellularly with 0mmol l^{-1} K^+ ASW containing various concentrations of Na^+. Both currents were reduced in amplitude by a reduction of the extracellular [Na^+] and reversed their polarity in 0mmol l^{-1} Na^+ ASW (Fig. 5A). The reversal potential for the fast transient current was Nernstian, exhibiting a mean slope of 54mV per decade (Fig. 5B) (*N*=5). The reversal potential for the non-inactivating current was difficult to

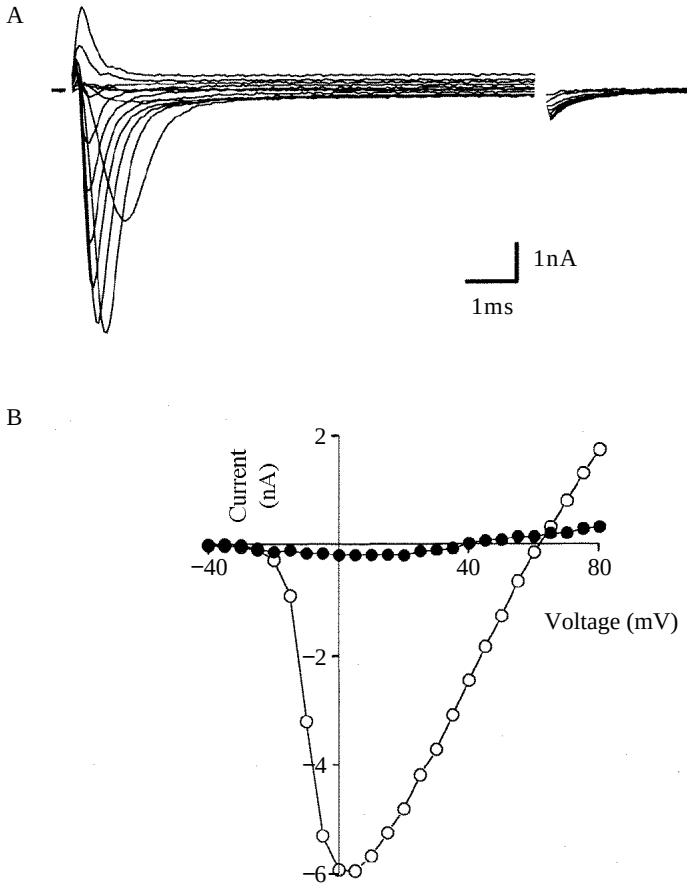

Fig. 4. Inward currents. (A) A family of inward currents recorded from a cell isolated from the brain of *Bdelloura candida*. The cell was clamped at −70mV and currents were evoked by 5mV voltage steps, starting at −40mV. Inward current consisted of a fast transient current that decayed to a steady-state phase. Bathing medium, K^+-free ASW; pipette solution, CsCl patch saline. (B) Current–voltage relationships of the peak transient (○) and steady-state (●) currents shown in A. Steady-state currents were measured at the end of the voltage step.

determine with any precision in the reduced-Na^+ salines, but it was clearly a function of extracellular [Na^+]. The amplitudes of both of the transient and steady-state inward currents, together with their activation thresholds, were independent of the extracellular [Ca^{2+}]. When the Ca^{2+} in the perfusate (66mmoll^{-1} Na^+) was replaced by Cd^{2+}, the amplitudes of the two currents were reduced only very slightly (*N*=5), indicating that neither was dependent on Ca^{2+} or blocked by Cd^{2+}.

The fast transient inward current inactivated in a voltage-dependent manner. Twin-pulse experiments designed to measure the coefficient of steady-state inactivation (H_∞), using 50ms pre-pulses, showed that the current evoked by a +10mV test pulse was half-inactivated at −20.6±6.8mV (*N*=5), while the non-inactivating current was unaffected (Fig. 6A). Reactivation of the fast transient current was studied by applying a 50ms

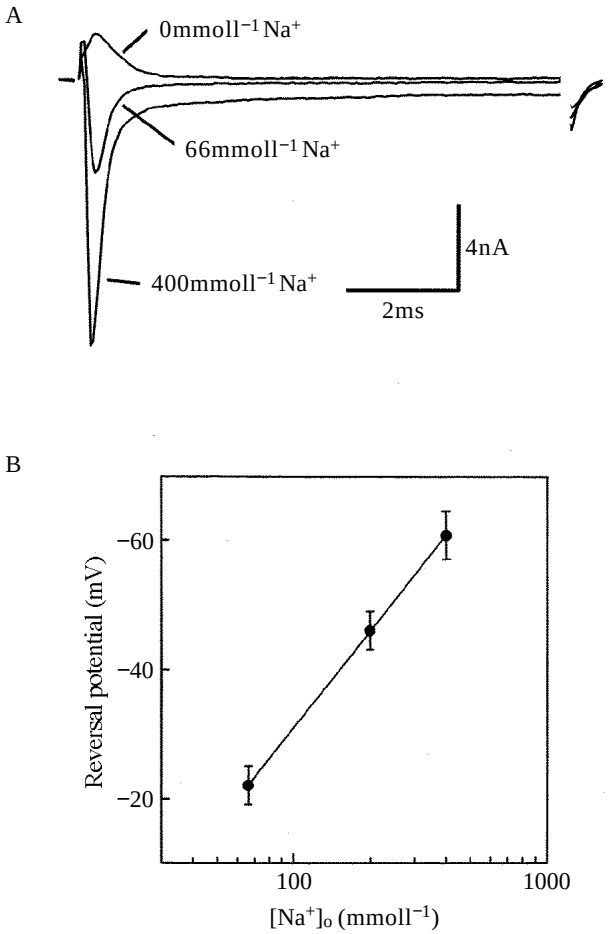

Fig. 5. Na^+-dependency of the fast transient and steady-state inward currents in *Bdelloura candida* neurones. (A) Effect of $[Na^+]_o$ on the inward currents evoked by a voltage step from −70mV to +10mV. Cells were bathed in 66mmol l^{-1} ASW then superfused with 400mmol l^{-1} Na^+ or 0mmol l^{-1} Na^+ ASW; pipette solution, CsCl patch solution (30mmol l^{-1} Na^+). Note that the currents reverse polarity when superfused with the Na^+-free saline. (B) Relationship between the reversal potential of the transient inward currents and $[Na^+]_o$. Data are presented as the mean ± S.E.M. for several trials from each of five cells. Bathing media, 66, 200 and 400mmol l^{-1} Na^+; pipette solution, CsCl patch solution (30mmol l^{-1} Na^+). The slope of the reversal potential was 54mV per decade.

conditioning pulse to +30mV, followed by repolarization to −70mV for various durations, and then by a test pulse to +10mV. The current was half-reactivated in 1.1±0.1ms (*N*=7) (Fig. 6B).

Both components of the Na^+ current were sensitive to several standard Na^+ channel blockers. In 400mmol l^{-1} Na^+, 0mmol l^{-1} K^+ ASW, both Na^+ currents were blocked completely by 100 μmol l^{-1} TTX, and the block was reversed when the cells were washed with TTX-free saline (Fig. 7A). To characterize the TTX-sensitivity of the

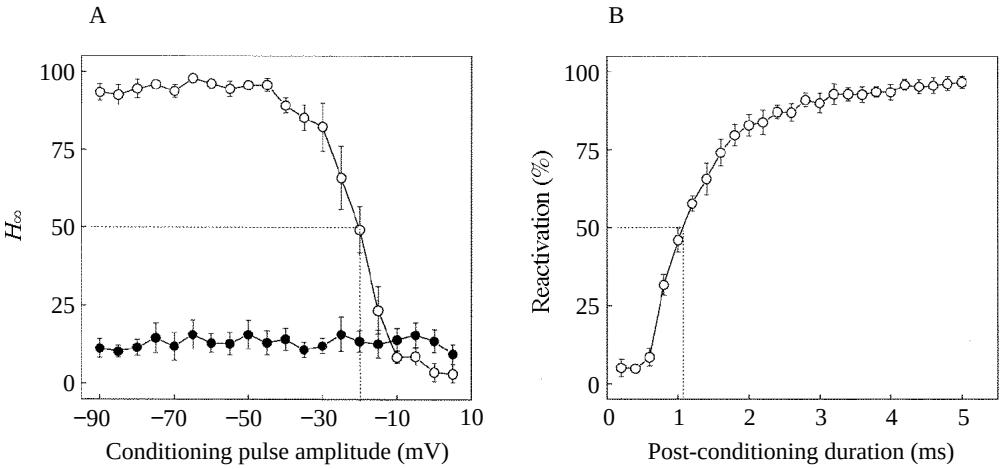

Fig. 6. Inactivation and reactivation of the inward currents in *Bdelloura candida* neurones. (A) A coefficient of inactivation (H_∞) plot for the transient (○) and steady-state (●) Na^+ currents. Currents generated by a test pulse to +10mV are plotted against the amplitude of a 50 ms conditioning pulse from the holding potential of −70mV. Data are presented as the mean ± S.E.M. (*N*=6 cells). Current amplitude is given as a percentage of the maximal evoked current at +10mV for each cell. The dotted lines mark the conditoning pulse amplitude that produced half-inactivation (−20.6mV). (B) Reactivation of the transient inward current. Following a 50ms pre-pulse to +30mV, the membrane potential was returned to the holding potential (−70mV) for a variable interval (the post-conditioning duration), then the membrane potential was stepped to +10mV. Here the amplitude of the current recorded at +10mV (expressed as a percentage of the evoked current after complete reactivation) is plotted against the post-conditioning duration. Data are presented as the mean ± S.E.M. (*N*=7 cells). Note that the current reactivates very quickly, consistent with the ability of these cells to spike at very high frequencies. The dotted lines show the time required for half-reactivation.

currents better, cells were perfused with 66mmol l^{-1} Na^+ ASW containing various concentrations of TTX. The fast transient current was blocked with an IC_{50} of 95nmol l^{-1} (49–190nmol l^{-1}, 95% confidence interval, *N*=6) (Fig. 7B). Block of the non-inactivating current was not significantly different from that of the inactivating current, but was difficult to quantify owing to its smaller size (data not shown). The fast transient current was reduced by 69±7% (*N*=3) by 100 μmol l^{-1} saxitoxin, which blocked the non-inactivating current in a similar manner. Lidocaine (1mmol l^{-1}) reduced both currents by roughly 50% (*N*=5). When the Na^+ currents were evoked from a holding potential of −70mV, the lidocaine-induced block was neither use- nor voltage-dependent. However, at more negative holding potentials, the cells became unstable and ultimately ruptured, precluding any attempts to relieve any pre-existing use- or voltage-dependent lidocaine block.

Sodium channel gating in higher animals is modified by a variety of lipophilic toxins and peptidergic venoms. Gating of the fast transient Na^+ current was not altered by veratridine at concentrations of 10–200 μmol l^{-1}. Venoms from the scorpions *Leiurus quinquestriatus* and *Centruroides sculpturatus* (25–500 μg ml^{-1}) and anemone toxin

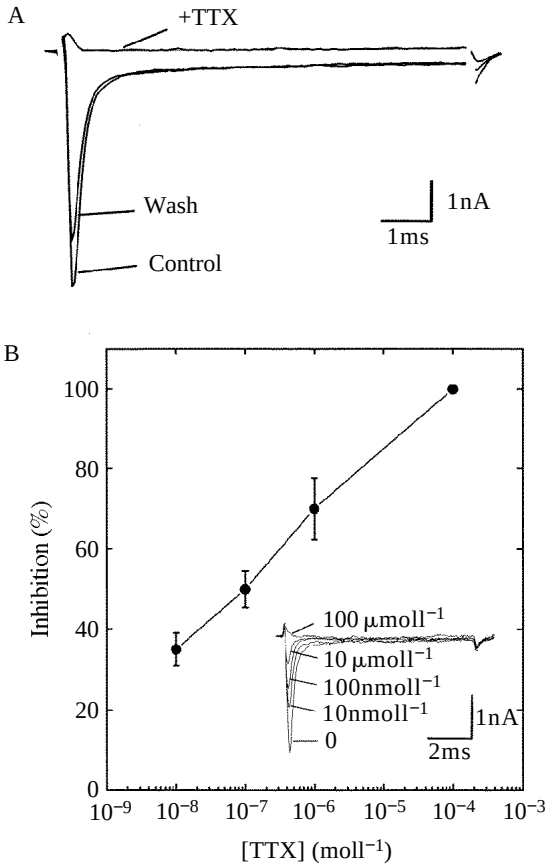

Fig. 7. TTX sensitivity of the Na^+ current in a representative *Bdelloura candida* neurone. (A) The Na^+ current was evoked by a voltage step from the holding potential of −70mV to +10mV. Superfusion of the cell with 100 $\mu mol\,l^{-1}$ TTX blocked both the transient and steady-state currents. This block was reversed rapidly upon washing the cell with TTX-free saline. Bathing medium, 400mmol l^{-1} Na^+ ASW; pipette solution, CsCl patch solution. (B) Dose–response curve for the action of TTX. The transient Na^+ current was blocked in a dose-dependent manner by TTX with an IC_{50} of 95nmol l^{-1} and 95% confidence intervals of 49–190nmol l^{-1}. In this reduced-Na^+ saline, the steady-state Na^+ current was lost in the background noise, and was not analysed for its TTX sensitivity. Data are presented as means ± S.E.M. (*N*=6 cells). Currents recorded from a single cell in the presence of increasing TTX concentrations are shown in the inset. Voltage paradigm as for A. Bathing medium, 66mmol l^{-1} Na^+ ASW; pipette solution, CsCl patch solution.

ATX-II (1 $\mu mol\,l^{-1}$) did not alter the activation or inactivation processes. However, each of these venoms and toxins accelerated the irreversible run-down of the Na^+ current.

To determine whether the fast transient current has an inactivation mechanism similar to that in higher animals, we examined the ability of proteases and oxidation to alter inactivation. The patch pipette was filled with a CsCl patch solution containing either trypsin (Sigma type IX, 0.1 or 1mg ml^{-1}) or Pronase E (2 or 8mg ml^{-1}), and the amplitude and kinetics of the Na^+ currents were examined with time. The fast transient

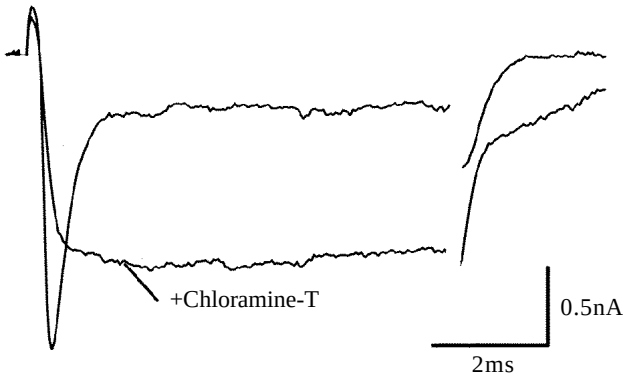

Fig. 8. Chloramine-T removes inactivation of the Na^+ current in *Bdelloura candida* neurones. The Na^+ current was evoked from a holding potential of −70mV by a step to +10mV. Bathing medium, 100mmol l^{-1} Na^+ ASW; pipette solution, CsCl patch solution. Superfusion with chloramine-T (1mmol l^{-1}) in 100mmol l^{-1} Na^+ ASW resulted in a complete loss of inactivation within 10s of bath application. Lower concentrations were less effective (not shown).

current decreased in amplitude and was lost within 30min at the lower enzyme concentrations (*N*=3) and within 5–10min at the higher concentrations (*N*=3 each). During this period, no change in the time course of the current and, specifically, the inactivating phase of the current, was observed. However, when cells were treated with 1 mmol l^{-1} chloramine-T, Na^+ current activation was slowed and inactivation was removed within 10s (Fig. 8) (*N*=3).

Ca^{2+} currents

In cells lacking a Na^+ current, or cells in which the Na^+ current had been blocked, a second, slower inward current was evident. This current was present in almost every cell examined but, in the majority of the cells, it was too small to work with. In the remaining 10–20% of the 15–30 μm diameter cells (Fig. 9A), the current was sufficiently large to be suitable for further study. This current activated at −21.2±6.3mV, reached maximum current amplitude at +5.0±4.0mV (Fig. 9B) and reversed between +50 and +55mV. The current had a pronounced inward tail (Fig. 9A) that decayed as a single exponential. In early studies, when the pipette saline contained 10mmol l^{-1} Hepes (pH7.0), the current decreased irreversibly in a time-dependent manner. This run-down was not prevented by including reagents such as ATP, GTP, NAD, NADH, NADP, NADPH, dithiothreitol, glutathione, cyclic AMP or phorbol esters in the pipette. However, when the pH buffering capacity of the pipette solution was increased and the pH was adjusted to 7.3, this current was stable for at least 30min. The pharmacological manipulations described below were all conducted under these stable conditions.

This inward current inactivated in a voltage-dependent manner. Twin-pulse experiments (50ms pre-pulses) showed that the current evoked by a test pulse to +10mV was half-inactivated at −40.0±3.5mV (*N*=3). Only one component of current was observed in these inactivation studies.

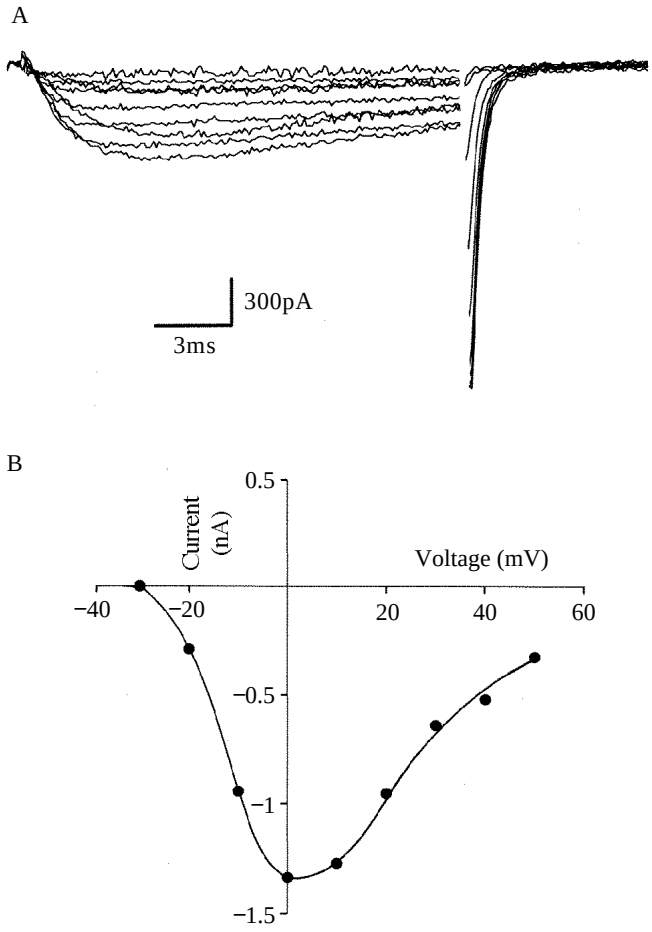

Fig. 9. (A) A family of Ca^{2+}-dependent inward currents recorded from a non-spiking cell from the brain of *Bdelloura candida*. The cell was clamped at a holding potential of −70mV. Currents were evoked by 10mV voltage steps from −30mV, applied at 10s intervals. These records represent the average of three trials at each voltage level. Bathing medium, 30mmol l^{-1} Ca^{2+} ASW; pipette solution, CsCl patch saline. (B) Current–voltage relationship of the peak inward currents shown in A.

This inward current was carried by Ca^{2+} since the current evoked by a test pulse to +10mV was reduced in amplitude when cells were perfused with 0mmol l^{-1} Ca^{2+} ASW. When the cells were bathed in 30mmol l^{-1} Ca^{2+} ASW, the amplitude of this inward current increased and its reversal potential shifted to the right. The threshold for activation, the time course of the current and its steady-state inactivation (H_∞) were not affected by changes in $[Ca^{2+}]_o$. Because of the relatively low signal-to-noise ratio of this current in 10mmol l^{-1} Ca^{2+} ASW, the remaining studies were carried out using 30mmol l^{-1} Ca^{2+} ASW.

The ion selectivity of the channels carrying this current was determined by perfusing the cells with 30mmol l^{-1} Ca^{2+}, Ba^{2+} or Sr^{2+} ASW (in random order) and evoking the

current with a test pulse to +10mV. The ion selectivity was $Ca^{2+} > Ba^{2+} \approx Sr^{2+}$, with the Ba^{2+} and Sr^{2+} current amplitudes being 50% of the Ca^{2+} current amplitude. This Ca^{2+} current was relatively insensitive to standard Ca^{2+} channel blockers. The Ca^{2+} current evoked by test pulses to +10mV in 30mmol l^{-1} Ca^{2+} ASW was reduced when the cells were perfused with 30mmol l^{-1} Ca^{2+} ASW containing 10mmol l^{-1} Co^{2+} (51.3±15 % block, *N*=3) or 10mmol l^{-1} Cd^{2+} (54.0±10.4% block, *N*=3). When the cells were perfused with 0mmol l^{-1} Ca^{2+} ASW containing 10mmol l^{-1} Co^{2+}, the inward current was blocked completely and a slow, voltage-dependent, outward current developed. This block was reversible upon perfusion of the cells with Co^{2+}-free 30mmol l^{-1} Ca^{2+} ASW. The Ca^{2+} current evoked by test pulses to +10mV was reduced by 10 μmol l^{-1} nifedipine (15.3±10.2% block, *N*=3), 10 μmol l^{-1} verapamil (31.0±19.9% block, *N*=3) and 10 μmol l^{-1} ω-conotoxin (17.3±6.8% block, *N*=3). The Ca^{2+} current was reduced by less than 5% when the cells were externally perfused with 30mmol l^{-1} Ca^{2+} ASW containing 0.1% ethanol, the carrier used for many of these agents.

Outward currents

Outward currents were examined in cells that lacked inward current altogether, and in cells where the inward currents were blocked. The resulting outward currents had a variety of waveforms and voltage dependencies. A transient component was almost always present, as was an apparently steady-state component, but the relative proportion of each and the time course of the decay of the transient component varied considerably from cell to cell. The threshold for activation of outward currents ranged from −35mV to −10mV. Time constants for inactivation of the outward currents varied widely: in 22 of 37 cells examined, inactivation during a test pulse to +70mV could be fitted with a single exponential whose time constant ranged from 10.7 to 104ms (mean 47.9±35.9ms); in the remaining 15 cells, outward current decay under the same conditions was best fitted by a double exponential with time constants of 0.83–11.8ms (mean 4.34±2.72ms) for the fast component and 10.0–74.6ms (mean 35.8±16.9ms) for the slower one. The steady-state components of outward current invariably activated at potentials more negative than those required to inactivate the transient components completely. Because of this overlap, the activation potentials of the steady-state components could not be determined accurately. Data from two cells, one with a significant fast transient component, the other with no fast transient current, are shown in Fig. 10. The activation potentials of outward currents in these cells are at the more negative end of the range encountered in this study.

Twin-pulse experiments designed to measure the coefficient of steady-state inactivation (H_∞) showed that the current evoked by a test pulse to +70mV was half-inactivated by 100ms pre-pulses to as little as −55mV in some cells and to as much as −10mV in others. The waveforms of the inactivating components of outward current were best revealed by subtracting the current remaining after complete inactivation of the transient component from the total outward current for a given cell (Fig. 10C,F).

These voltage-gated outward currents appeared to be carried exclusively by K^+. Twin-pulse experiments with a 6ms prepulse to +70mV were used to characterize the reversal of tail currents in cells devoid of inward currents. With KCl in the pipette, the tail currents

in cells bathed in ASW containing either 10mmol l^{-1} (E_K−95mV) or 130mmol l^{-1} (E_K−30mV) KCl reversed at −82.0±2.2mV (N=4) or −33.0±0.0mV (N=4), respectively. The reversal potentials for tail currents recorded in these two media did not

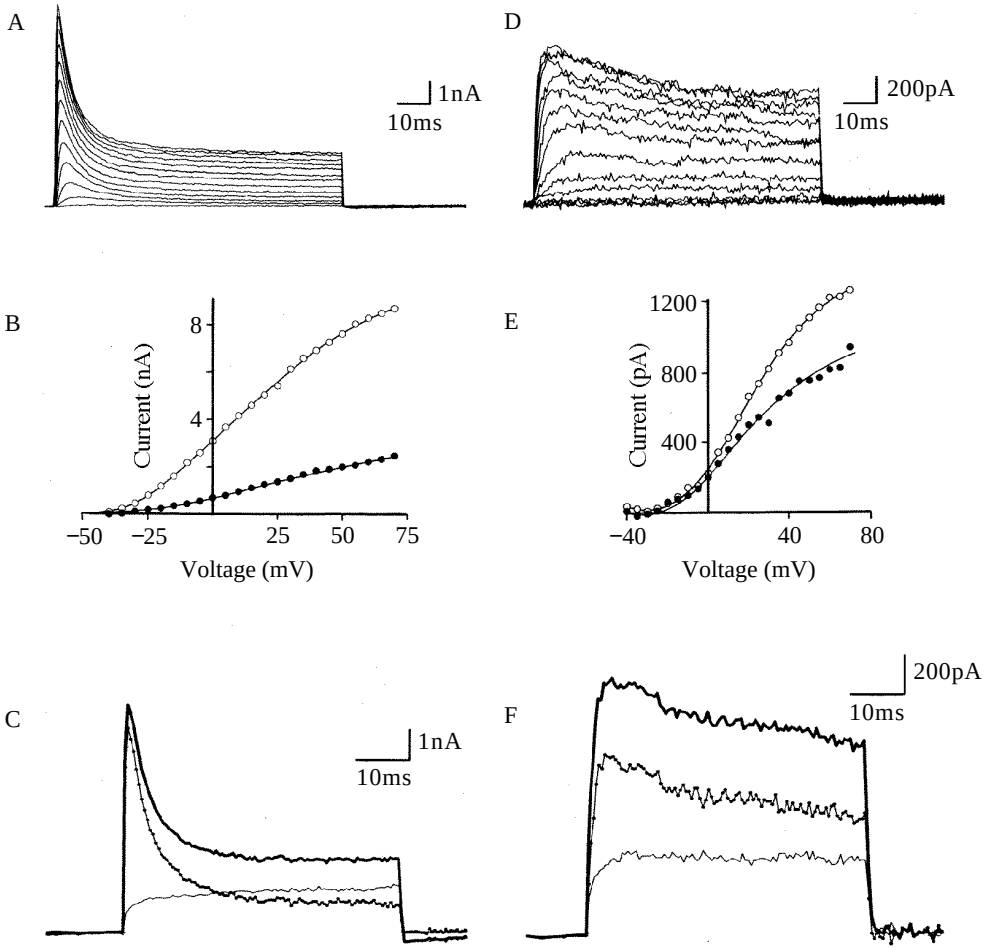

Fig. 10. Diversity of voltage-gated K^+ currents in cells from *Bdelloura candida* brains. (A) A family of outward currents from a cell that expressed a large amount of fast-inactivating outward current. The cell was clamped at a holding potential of −70mV and currents were evoked by 10mV voltage steps from −40mV. (B) Current–voltage relationship of the peak current (○) and current remaining at the end of the voltage step (●) for the currents shown in A. Current recorded at intermediate voltage steps, but not shown in A, are included in this plot. (C) Transient and non-inactivating components of these currents, revealed by selective inactivation of the transient component. The transient current (small dots) was generated by subtracting the residual non-inactivating current (fine line) from the total outward current evoked by a test pulse to +70mV (bold line). The residual non-inactivating current was generated by a voltage step to +70mV, following a 500ms preconditioning pulse to −40mV, which inactivated the transient current. (D–F) The same as A–C for a family of currents from a cell lacking any fast transient outward current. Bathing media in all cases, ASW; pipette solutions, KCl patch saline.

change (85.0±7.7mV, N=5, and −30.0±5.9mV, N=5mV, respectively) when the pipettes were filled with $KMeSO_3$. This was true even when the same cell was patched sequentially with electrodes containing the two different patch solutions, indicating that there was no contribution by Cl^- to the outward currents.

Cells that expressed Ca^{2+} currents were voltage-clamped in 10mmol l^{-1} Ca^{2+} ASW and then perfused with 0mmol l^{-1} Ca^{2+} ASW. Subtraction of the currents evoked under these two conditions demonstrated two different phenomena. At test potentials between −10 and +30mV, the subtraction current consisted only of the underlying Ca^{2+} current. Above +40mV, where the Ca^{2+} current was near its apparent reversal potential, the subtraction current increased in amplitude in a voltage-dependent manner. This behaviour, which would not be expected if a significant component of the outward current were Ca^{2+}-dependent, suggests that outward currents are actually blocked by Ca^{2+}.

The transient components of the outward currents were blocked selectively by 10mmol l^{-1} 4-aminopyridine (4-AP). Tetraethylammonium (TEA^+, 10mmol l^{-1}) was ineffective on most cells but, when it blocked, it selectively blocked the non-inactivating K^+ current. Ba^{2+} (10mmol l^{-1}) had little effect on any of the K^+ currents, blocking to the same degree as extracellular Ca^{2+} appeared to block. In cells that lacked any apparent inward current, Cd^{2+} blocked outward currents as effectively as either TEA^+ or 4-AP.

Close scrutiny of the distribution of K^+ currents among different cells revealed that specific components of outward current were co-expressed with specific inward currents. Approximately 60–70% of the cells examined possessed outward current. Those cells that lacked any inward current, and those that had a Ca^{2+} current but no Na^+ currents, possessed only the slowly inactivating (time constant t>20ms) outward current and a steady-state current. Cells that had Na^+ currents also possessed fast transient outward currents (t<5ms).

Discussion

The brains of platyhelminths are discrete structures that are located rostrally and, in this way, resemble the brains of higher organisms. However, the remainder of the animal is innervated by several plexuses or nerve nets that resemble those of cnidarians. Many neurones within these nerve nets originate in the brain and extend the length of the animal (Koopowitz, 1989), requiring that the animals possess the capability for producing action potentials. The presence of action potentials in these organisms has been well documented (Keenan and Koopowitz, 1981, 1984; Solon and Koopowitz, 1982; Koopowitz, 1989), but characterization of the underlying currents has been severely hampered by the limited number and small size of the cells and by the problems incurred when trying to infer details about ionic currents on the basis of the sometimes subtle changes in the waveform of action potential produced by ionic and pharmacological manipulations.

Clearly, cells from *Bdelloura candida* possess a full complement of ionic currents, including at least one Na^+ current, a Ca^{2+} current and several K^+ currents. There are two distinct components of the inward Na^+ current; one a fast transient current, the other non-inactivating (Fig. 4). Both components of the Na^+ current have the same activation

potentials, and, so far as can be ascertained, the same Na^+- (Fig. 5) and TTX-dependencies (Fig. 7), suggesting that both components of Na^+ current might be carried by the same channel. However, in the absence of single-channel recordings, or a specific blocker for either component of the inward current, one cannot determine whether there are two pharmacologically similar types of Na^+ channel or only one with both inactivated and non-inactivated open states, as has been reported for squid (Keynes, 1991), barnacle (Davis and Stuart, 1988) and the vertebrate brain (French *et al.* 1990). It is reasonable to assume that the fast transient component of inward current in these cells is responsible for the depolarizing phase of the TTX-sensitive Na^+ spikes reported by Keenan and Koopowitz (1981), whereas the steady-state component is probably responsible for the Cd^{2+}-insensitive plateau phase reported by the same authors.

The activation potential of the Na^+ current (-16mV) is very depolarized compared with that typically found for Na^+ currents, but is very similar to that of Na^+ currents in cnidarians (Anderson, 1987) and is consistent with the very depolarized activation potential of ionic currents in a variety of lower organisms (Oertel *et al.* 1977; Naitoh, 1982; Anderson and McKay, 1987; Dubas *et al.* 1988; Dunlap *et al.* 1987; Holman and Anderson, 1991). The kinetics of activation and inactivation (Fig. 6B) of the transient component are notably fast, as is its recovery from inactivation (Fig. 6B), properties that are consistent with, and essential for, the very high spike frequency (>300Hz) exhibited by these cells. Curiously, the inactivation process could be modified by oxidation but not by proteolytic digestion, indicating that, although the flatworm Na^+ channel can be converted to an inactivated state, it lacks the proteolytic cleavage sites found in higher animals.

Pharmacologically, the flatworm Na^+ current resembles other neuronal Na^+ currents in that the Na^+ currents in *Bdelloura candida* were sensitive to TTX (IC_{50}=95nmoll^{-1}) (Fig. 7), saxitoxin and lidocaine. However, inactivation of the fast transient component of the Na^+ current was not obviously affected by veratridine, scorpion venom and ATX-II toxin, agents that normally block Na^+ channel inactivation. The fact that the run-down of the current was accelerated by these agents suggests that the channels might bear the appropriate receptors for these drugs and venoms, but that binding is not manifested in the same physiological changes that occur in higher animals.

The whole question of the TTX-sensitivity of flatworm Na^+ currents is important from the viewpoint of Na^+ channel evolution. The phylum Platyhelminthes forms the base of most phylogenetic trees for the animal kingdom, whereas the Cnidaria form a separate line that presumably evolved from a common stem group. As noted earlier, Na^+ currents in cnidarians are completely insensitive to TTX (Anderson, 1987; Hille, 1992), and previous studies (Keenan and Koopowitz, 1981) had reported that flatworms possess both TTX-sensitive and TTX-insensitive Na^+ currents. The presence of both TTX-sensitive and TTX-insensitive Na^+ currents in flatworms might suggest that the TTX receptor evolved in this phylum, possibly from a channel similar to that found in cnidarians. However, the presence of only TTX-sensitive currents would imply that the TTX receptor evolved prior to evolution of the Platyhelminthes, presumably after the separation of the Cnidaria from the invertebrate lineage. All Na^+ currents examined in this study were TTX-sensitive and, although other species should be studied to ensure that TTX-

sensitivity is the norm for the phylum, this finding suggests that the TTX receptor evolved after separation of the Cnidaria from the protostome/deuterostome line, prior to the appearance of the Platyhelminthes. The TTX-insensitive action potentials reported by Keenan and Koopowitz (1981) may merely reflect the relatively low TTX-sensitivity of the flatworm Na^+ channels (IC_{50}=95nmol l^{-1}) combined with the low concentrations used in that study (10nmol l^{-1}).

These cells apparently possess a single type of Ca^{2+} current (Fig. 9). Its selectivity, $Ca^{2+} > Ba^{2+} \approx Sr^{2+}$, is similar to that of muscles from the sea anemone *Calliactis tricolor* (Holman and Anderson, 1991) and the hydromedusan jellyfish *Polyorchis pennicilatus* (Przysiezniak and Spencer, 1992), and is generally in keeping with the typical ionic selectivity of Ca^{2+} currents in lower organisms. The low sensitivity of the Ca^{2+} current to the blockers tested indicates that it is not of the T-, N- or L-type. Its similarity to P-type Ca^{2+} currents was not evaluated.

Outward currents in these cells are carried exclusively by K^+ and, although they form an apparently heterogeneous family, they can be dissected into three distinct types; fast transient currents with a time constant of inactivation of less than 5ms, more slowly inactivating (t>20ms) transient currents, and steady-state currents. Pharmacologically, these outward currents are not remarkable. The sensitivity of the transient outward currents to 4-AP is consistent with their being A-type currents (Hille, 1992), and the weak TEA^+-sensitivity of the steady-state currents is compatible with the previous finding (Solon and Koopowitz, 1982; Keenan and Koopowitz, 1984) that TEA^+ must be injected intracellularly to block K^+ currents in cells *in vivo*. The co-expression of the rapidly inactivating K^+ current and Na^+ currents implies a functional connection between the two and may be a mechanism to enable the cells to produce action potentials at high frequencies. The diversity and pharmacology of outward currents in these cells is similar to that recently reported for cells of the parasitic nematode *Ascaris suum* (Martin *et al.* 1992) and is reminiscent of that of outward currents in neurones from the hydrozoan jellyfish *Aglantha digitale* (Meech and Mackie, 1993).

Action potentials recorded intracellularly from neurones in the brain of the free-living flatworm *Notoplana acticola* are characterized by a marked hyperpolarizing afterpotential (Keenan and Koopowitz, 1984) that is abolished by removal of extracellular Ca^{2+} or substitution of Ba^{2+} for Ca^{2+}. This finding was interpreted as indicating that those cells possessed a calcium-activated potassium conductance (I_{KCa}). No evidence for such a current was obtained in this study. However, the high levels of Ca^{2+} buffering used in these experiments may have prevented intracellular Ca^{2+} concentrations attaining the levels required for activation of I_{KCa}.

One of the most notable findings was that so few cells possess voltage-activated inward currents, and only 5% of the cells examined produced action potentials. This number could not be raised by selecting cells with distinct processes or by the use of various culture conditions or conditioning factors. Inherent in this problem is the identity of the cells examined in this study. The variety of cell morphologies observed in these cultures (Fig. 1) is consistent with that of neurones in intact brains (Koopowitz, 1989). Flatworm brains also contain what has been described as glia (Golubev, 1988), which form well-developed sheaths that enwrap the neurones (Koopowitz, 1989). Our cell cultures could,

therefore, consist of both cell types and the two could be indistinguishable under the conditions employed. The smallest cells present in the cultures (5–15 μm) produced no inward currents at all, suggesting that they are completely inexcitable. Thus, one might argue that they are most likely to be glial cells. However, only 5% of the larger (15–30 μm) cells produced action potentials, suggesting either that some of the larger cells are also glial cells or that many neurones are non-spiking. Non-spiking cells are indeed quite common in the brains of free-living platyhelminths (Koopowitz, 1989), so their presence in these cultures is not altogether unexpected. However, it is very difficult to determine the proportion of non-spiking neurones in intact brains. The bulk of intracellular recordings from intact brains has purposefully been made from regions where spiking cells are more commonly encountered (L. Keenan, personal communication). Furthermore, since one criterion for a successful impalement and, therefore, a reason to continue recording from a given cell, is the presence of action potentials, there would be an additional bias towards an overestimation of the relative abundance of spiking neurones. Nevertheless, most of the motor neurones that innervate distant muscles and plexuses originate in the brain (Koopowitz, 1989), so they presumably produce action potentials to achieve long-distance propagation. The explanation that we favour to explain the low abundance of spiking cells in our preparations is that the majority of neuronal somata in these animals do not possess voltage-activated Na^+ currents, as is the case with many invertebrates (Brismar and Gilly, 1987), and that the electrical excitability of the motor neurones is restricted to their axons. Although many of our cells did develop processes (Fig. 1), those processes were all relatively short and may not have developed the true characteristics of axons. Other explanations notwithstanding, the low percentage of cells with measurable currents is a serious limitation to further work on these organisms, since large numbers of cells must be screened before any definitive conclusions are made.

This work was supported by a grant from the National Science Foundation (BNS 9109155) to P.A.V.A.

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