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PROPERTIES AND PHARMACOLOGY OF A TTX-INSENSITIVE Na^+ CURRENT IN NEURONES OF THE JELLYFISH *CYANEA CAPILLATA*

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SUMMARY

1. Neurones of the motor nerve net of the jellyfish *Cyanea capillata* were axotomized, and voltage-clamped using the whole-cell configuration of the patch-clamp technique.

2. Outward currents were blocked by a combination of extracellular 4-aminopyridine (4-AP) and intracellular Cs^+, tetraethylammonium (TEA^+) and 4-AP.

3. Under normal conditions, the inward current consisted of a fast, transient current which could be abolished by removal of extracellular Na^+ and whose reversal potential was dependent on the extracellular Na^+ concentration.

4. This current was completely insensitive to tetrodotoxin (TTX), saxitoxin (STX) and conotoxin GIIIA but could be blocked by extracellular Cd^{2+}, lidocaine, W7 [*N*-(6 aminohexyl)-5-chloro-1-napthalenesulphonamide] and verapamil.

5. Inactivation was voltage-dependent with a V_h of -15 mV, and was unaffected by veratridine, batrachotoxin (BTX), sea anemone toxin and scorpion (*Leiurus*) venom. Reactivation required repolarization to a negative membrane potential for 12 ms for half-maximal reactivation.

6. In the absence of extracellular Na^+ no inward current was visible unless $[Ca^{2+}]_o$ was elevated. In Na^+-free, 95 mmol l^{-1} Ca^{2+} saline, a slightly slower, inward current was recorded. This current is believed to be the Ca^{2+} current that underlies synaptic transmission.

7. These findings are discussed with reference to synaptic transmission in these cells and the evolution of ion channels.

INTRODUCTION

Action potentials in neurones and other electrically excitable cells are produced by the movements of ions through discrete, voltage-gated ion channels. While Ca^{2+}-dependent action potentials are common in certain muscles and in the somata of various invertebrate neurones, particularly those in molluscs (reviewed by Adams, Smith & Thompson, 1980), the axons of most neurones are designed for rapid propagation and here Na^+ channels predominate. A great deal is now known about

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the biophysics, pharmacology and, more recently, the structure and composition of Na^+ channels (Armstrong, 1981; Catterall, Gonoi & Costa, 1985; Ellisman, Agnew, Miller & Levinson, 1982; Hille, 1984) and the overall picture suggests that there has been a high degree of conservation of the Na^+ channel through evolution. However, very little is known about the properties of Na^+ channels in the primitive animals in which axonal Na^+ channels are thought to have evolved (Hille, 1984), primarily because these animals offer very few preparations where Na^+ currents can be examined in sufficient detail.

Coelenterates are the most primitive phylum to possess a recognizable nervous system. More relevant here, they are the most primitive animals to produce Na^+-dependent action potentials (Anderson & Schwab, 1982) and can thus provide useful information about the properties of the primitive Na^+ channel.

The motor nerve net of the scyphomedusan jellyfish *Cyanea capillata* is composed of relatively large, bipolar neurones (Anderson & Schwab, 1981) which are connected by bidirectional, excitatory chemical synapses (Anderson, 1985). These neurones are organized into a two-dimensional plexus which serves to transmit activity from marginal pacemakers to the swimming musculature. Conventional microelectrode recordings (Anderson & Schwab, 1983, 1984) have shown that these neurones produce fast, overshooting, Na^+-dependent action potentials. One remarkable feature of the Na^+-dependent action potentials in cnidarians is that they are completely insensitive to the classical Na^+ channel blocker tetrodotoxin (TTX) (Anderson & Schwab, 1983, 1984). Neurones of the motor nerve net can be exposed (Anderson & Schwab, 1984) and current-clamp recordings have been made (Anderson, 1985) with the whole-cell configuration of the patch-clamp technique (Hamill *et al.* 1981). This paper describes the results of a voltage-clamp study of these cells with particular reference to the inward currents.

MATERIALS AND METHODS

The preparation

Specimens of *Cyanea capillata* were obtained either from the intracoastal waterway in the vicinity of the Whitney Laboratory or from stocks cultured in the laboratory.

Neurones of the motor nerve net were exposed by brief oxidation of the surface of pieces of perirhopalial tissue (Anderson & Schwab, 1981, 1984). Briefly, the pieces were pinned to a layer of Sylgard (Dow Corning, Midland, MI) at the base of a Petri dish, using cactus spines (*Opuntia* sp.), and bathed for 3–5 s with 0·05 % (w/v) sodium hypochlorite in sea water. The tissue was quickly rinsed seven times with fresh sea water and bathed in *Cyanea* saline (Anderson & Schwab, 1984), supplemented with 2 mg ml^{-1} glucose and 2 % foetal calf serum (Gibco, Grand Island, NY). After 1 h at 9 °C, the epithelium had degenerated and was peeled off using fine forceps.

This procedure produced preparations which varied from essentially complete nerve nets consisting of several hundred neurones per piece of perirhopalial tissue to

others in which only a few neurones remained. The reason for the difference is unclear, but when only a few neurones remained, it was observed that most neurones had remained attached to the epithelial debris and were removed with it. It is unlikely that the neurones in any of these preparations were exposed to the oxidant since we have found, not surprisingly, that direct exposure of the neurones to hypochlorite causes rapid cell death. Previous work (Anderson & Schwab, 1984) has shown that the basic properties (resting potential, action potential amplitude, rate of depolarization and duration) of intact neurones exposed in this manner and bathed in this saline are the same as those of neurones in the intact tissue.

Neurones in this nerve net are bipolar. The soma forms an obvious 10–20 μm swelling of the otherwise smooth, relatively straight axon, which has a diameter of 2–5 μm and can be as long as 1 cm. Clearly, neurones with this type of morphology could not be adequately space-clamped *in situ*. For these recordings, a space-clamp was achieved either by selecting cells that had lost their axons during cell exposure or by selectively axotomizing intact cells. Attempts to cut the axons with microelectrodes or other sharp instruments usually failed, primarily because the underlying mesogloea lacked the necessary resilience. Instead, axotomy was achieved by placing a small ($<$50 μm diameter), saline-filled pipette over the axon close to the soma and applying one or more large (50 V) and relatively long (100 ms) stimuli. The axons usually broke very readily and the severed ends retracted. Recordings could be made from these cells within 2 min indicating that the cut ends of the axons must have resealed very rapidly. Those recordings were no different from those obtained from cells which had lost their axons during exposure, indicating that the electrical properties of the cells had not been compromised by the electrical stimuli. The possibility that the cells' electrical properties were altered by axotomy (Pitman, Tweedle & Cohen, 1972) remains since, for the reasons outlined above, it was not possible to voltage-clamp the intact, exposed cells. However, recordings were frequently obtained within a few minutes of axotomy, indicating that any changes must have occurred extremely rapidly, far faster than has been reported in other preparations (Pitman *et al.* 1972) where changes typically occur with a time course of several days. Furthermore, the results of the voltage-clamp experiments described here are consistent with our knowledge of these cells from current-clamp experiments using intact *in situ* (Anderson & Schwab, 1983) or exposed cells (Anderson, 1985; Anderson & Schwab, 1984).

Recording techniques

Voltage-clamp recordings were obtained with the whole-cell configuration of the patch-clamp technique (Hamill *et al.* 1981). Patch pipettes were pulled from borosilicate glass (Boralux, Rochester, Scientific, Rochester, NY) and coated with a layer of cured Sylgard (Dow Corning, Midland, MI). These electrodes were not fire-polished after coating since better seals were obtained when fire-polishing was omitted. The pipette was filled with one of the solutions given in Table 1. With normal patch solution, pipettes had resistances of 3–5 MΩ when immersed in *Cyanea* saline.

Seals were obtained in the usual manner. Electrode capacitance was neutralized with circuitry in the amplifier (Dagan 8900). Seal formation was facilitated by applying a negative potential to the inside of the pipette. Seals with resistances of 1–10 GΩ could be obtained routinely under these conditions. If the pipettes were not coated with Sylgard, or fire-polished after coating, seals were usually in the range 100–500 MΩ. Attempts to 'clean' the surface of the cells enzymatically have so far proved unsuccessful.

Break-through into the intracellular configuration was achieved with negative pressure. Immediately upon break-through, series resistance error compensation was added with controls in the amplifier. This was done subjectively by adding sufficient compensation to reduce transient settling time to a minimum consistent with clamp stability. Subsequent measurements of series resistance indicated that from 40 to 60 % of series resistance was compensated for in this way. In the absence of any series resistance compensation, capacitive transients settled to 95 % of baseline within 0·8–1·2 ms, depending on the size of the cell fragments. After the addition of series resistance compensation, settling time decreased, on average by 30 %. Series resistance compensation was monitored throughout every recording and readjusted if necessary.

Voltage-clamp experiments were carried out, and the data digitized (80 kHz), stored and manipulated with an IBM AT computer equipped with pClamp Software (Axon Instruments, Burlingham, CA). Leakage and capacitive currents were removed from the current records either by adding currents generated by equal and opposite voltage steps or by scaling and subtracting currents generated by voltage steps one-third of the amplitude of those used to generate ionic currents. The latter were applied from a holding potential of −100 mV.

Solutions

The compositions of all solutions used in this study are given in Table 1. All extracellular solutions were adjusted to pH 7·4 with NaOH and HCl or, in the case of Na^+- and Cl^--free salines, with CsOH and methanesulphonic acid (Eastman), respectively. For isolation of inward currents, 4-aminopyridine (4-AP) was added to the saline directly, as were antagonists such as tetrodotoxin (TTX), saxitoxin (STX), batrachotoxin (BTX), verapamil, lidocaine, the calmodulin antagonist W7 [*N*-(6 aminohexyl)-5-chloro-1-naphthalenesulphonamide] and Cd^{2+}. W7 and verapamil were dissolved in ethanol prior to their addition to the recording solution. The final ethanol concentration never exceeded 2 % (v/v) and at this concentration ethanol had no noticeable effect on recorded currents. These antagonists were added to the saline as needed. Unless otherwise stated, all experiments on the inward currents in these cells were carried out in the absence of either TTX or STX.

The motor nerve net neurones in the exposed cell preparations used in this study were only loosely attached to the underlying mesogloea and it proved exceedingly difficult to exchange the bath contents or to perfuse the vicinity of the cell with a different solution without jeopardizing the recording. For this reason, the effects of channel blockers were assessed by examining their effect on normalized currents

Table 1. *Composition of solutions*

Salt	External solutions ($mmol\,l^{-1}$)				Patch solutions ($mmol\,l^{-1}$)	
	Saline*	Na^+-free	Ca^{2+}-free	Cl^--free	Normal	Cs^+/TEA^+
NaCl	390	—	399·5	—	—	30
KCl	13·4	13·4	13·4	—	140	140
$CaCl_2$	9·5	9·5	—	—	1	1
$MgCl_2$	24	24	24	—	—	—
$MgSO_4$	5	5	5	5	—	—
Choline chloride	41·5	41·5	41·5	—	—	—
Hepes	10	10	10	10	10	10
Sodium aspartate	—	—	—	390	—	—
TEACl	—	390	—	—	—	70
CsCl	—	—	—	—	—	70
Glucose	—	—	—	98·7	696	696
Potassium gluconate	—	—	—	13·4	—	—
Calcium gluconate	—	—	—	9·5	—	—
Magnesium gluconate	—	—	—	24	—	—
EGTA	—	—	—	—	11	11
4-AP	—	—	—	—	—	2

*Anderson & Schwab (1984).
TEA, tetraethylammonium, 4-AP, 4-aminopyridine.

recorded from different cells. Averaged data are expressed as the mean ± the standard error of the mean. All experiments were conducted at room temperature (22–25°C).

RESULTS

Neurones of the motor nerve net of *Cyanea* have a mean resting potential of −59 mV (Anderson & Schwab, 1983). The action potential, as recorded with a microelectrode (Anderson & Schwab, 1983) or a patch pipette in the current-clamp mode (Anderson, 1985) is a fast event which overshoots 0 mV, sometimes by as much as 35 mV. Its time to peak is of the order of 1 ms or less but its duration is very variable, primarily because of the large number of superimposed synaptic potentials. In the absence of synaptic activity, duration at half peak amplitude was typically 2–4 ms. The overall aim of this work was to examine the properties of the ionic current(s) responsible for the depolarizing phase of this action potential.

Total membrane currents

Total membrane current recorded under voltage-clamp from an exposed, axotomized neurone consisted of a fast, transient, inward current followed by a fairly complex outward current (Fig. 1A). Inward current activated at −15 mV, reached peak amplitude at +15 mV and reversed in the vicinity of +60 mV (Fig. 1B). Outward current usually activated at slightly more positive membrane potentials (−10 mV). For small voltage steps to less than +30 mV, outward current was steady-state but with voltage steps to more positive potentials the steady state current was

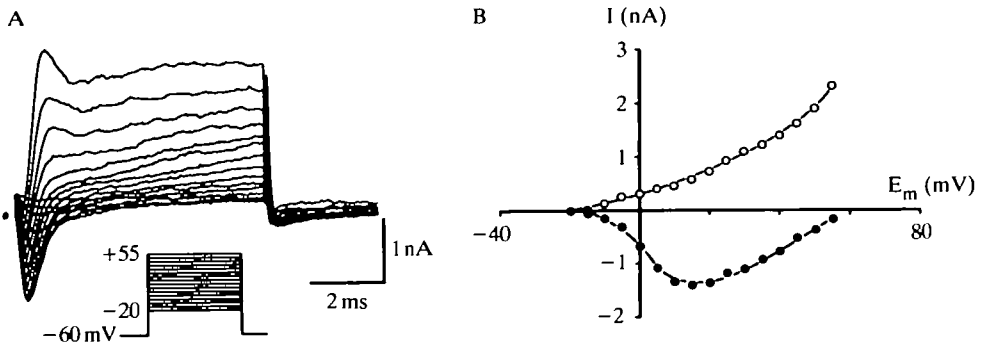

Fig. 1. (A) Total membrane currents recorded under voltage-clamp from an axotomized neurone in the motor nerve net of *Cyanea*. The voltage regime is shown as an inset; voltage steps increased in 5-mV increments. (B) Current/voltage plot for the transient inward (●) and steady-state outward currents (○) shown in A. The latter current amplitude was measured at the end of the voltage step. External medium, normal saline; internal medium, normal patch solution.

preceded by a relatively fast, transient component. Outward currents in these cells were not examined in any further detail except to note that all outward current could be blocked by a combination of extracellular 4-AP (7·5 mmol l^{-1}) and intracellular Cs^+ (70 mmol l^{-1}), TEA^+ (70 mmol l^{-1}) and 4-AP (2 mmol l^{-1}). Blockade occurred rapidly, usually within 1 min of break-through, leaving the inward current in isolation.

Inward currents

Inward current in these cells was dominated by a fast, transient current (Fig. 2A) which activated with a time constant of 500–800 μs, reaching peak amplitude within 1 ms. Inactivation was slower, having a time constant of 1·2–3·7 ms, but was rarely complete. Instead, a steady-state component usually remained. This inactivated very little during a voltage step (time constant of decay = 5–10 ms) and, at the end of the voltage step, a very obvious inward tail current occurred. The ratio of peak transient current amplitude to steady-state current amplitude was not constant but varied from cell to cell. The transient current was usually the dominant current, with the steady-state current appearing to differing degrees in the different cells. The transient current was rarely absent; it was more common to find cells that lacked the steady-state component.

Inward current activated at −10 to −20 mV (Fig. 2B), reached peak amplitude at close to +10 mV and reversed at around +60 mV (mean reversal potential in 390 mmol l^{-1} Na^+ = +62·9 ± 0·9 mV). Because inward current was dominated by the transient current which was small, relatively slow and had not yet developed its transient waveform when it first activated, it was not possible to distinguish between the activation potentials of the transient and steady-state currents.

Inactivation of the transient current was examined using the pre-pulse regime depicted as an inset of Fig. 3A. Cells were clamped at a holding potential of

−100 mV. Inward currents were generated by a test pulse to +10 mV. This was preceded by a 50 ms duration pre-pulse of variable amplitude. Pre-pulses to potentials more negative than −35 mV had no effect on inward current (Fig. 3A), but inward current was reduced by pre-pulses to potentials more positive than −35 mV and essentially abolished by pre-pulses to +10 mV. V_h, the mean voltage for half-inactivation of the inward current, was measured from this curve and for this cell was found to be −15 mV. This value was typical of that in all cells examined.

Inactivation could be reversed by the interposition of a hyperpolarizing step after the pre-pulse (Fig. 3B). For these experiments, cells were clamped at a holding potential of −100 mV and then depolarized with a pre-pulse to −20 mV followed, at a variable interval, by a test pulse to +10 mV during which current was recorded. In the interval between the pre-pulse and the test pulse, the post-conditioning interval, the cell was hyperpolarized to −100 mV for a variety of durations. A plot of post-conditioning duration against inward current amplitude (Fig. 3B) reveals that inactivation was partially reversed by as little as 5 ms at a hyperpolarized potential, with complete reversal requiring of the order of 50 ms. One-half reactivation was attained in 12 ms.

Because all recordings were obtained from axotomized cells, each of a different size with a different amount of axon attached, the amplitudes of the inward currents recorded from the different cells varied considerably. Frequently, recordings had to be terminated because the action potential could not be adequately clamped, as evidenced by the all-or-nothing appearance of the inward current at the activation potential of the inward current and, in such cases, this could usually be attributed to excess axonal membrane. To permit comparison of currents recorded from different cells under different conditions, recorded currents were normalized as current densities, on the basis of cell capacitance. Cell capacitance was determined from the capacitive transient recorded after break-through, before the addition of series

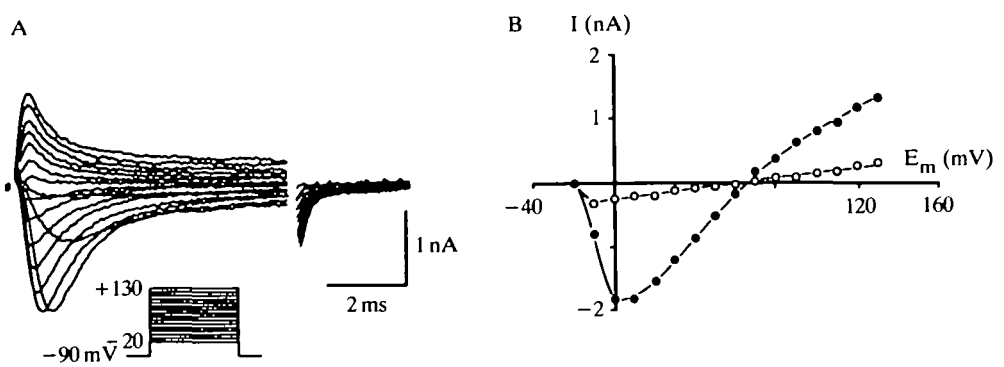

Fig. 2. (A) A family of inward currents recorded from a neurone from *Cyanea*. Voltage steps increased in 10-mV increments. Note that inward current consisted of a fast, transient current which partially decayed to a steady-state phase. External medium, saline with 7·5 mmol l^{-1} 4-aminopyridine (4-AP); internal medium, Cs^+/TEA^+ patch solution with 2 mmol l^{-1} 4-AP and 30 mmol l^{-1} Na^+. (B) Current/voltage relationships of the transient (●) and steady-state (○) currents shown in A. Steady-state current amplitudes were measured at the end of the voltage step.

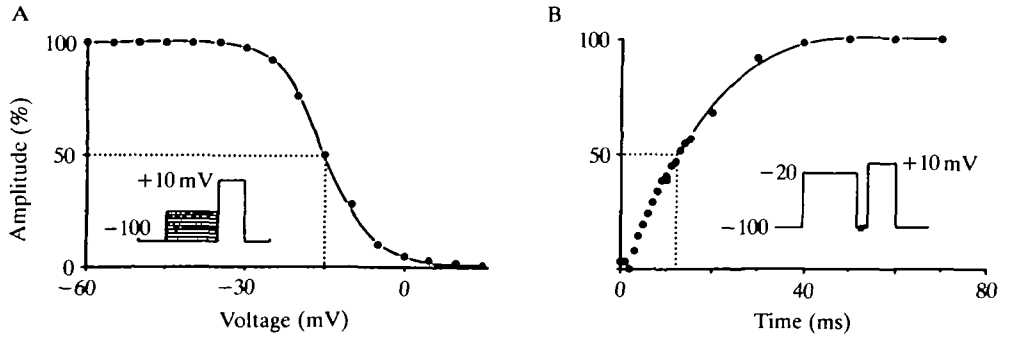

Fig. 3. Inactivation and reactivation of the transient inward current. (A) An H-infinity curve for one cell. The voltage regime is shown as an inset. Currents generated by a test pulse to +10 mV are plotted against the amplitude of a 50-ms pre-pulse from the holding potential of −100 mV. Each point represents the mean for several trials with the same cell. Current amplitude is given as a percentage of that measured in the absence of any pre-pulses. The dotted lines indicate V_h. (B) Reactivation of the inward current. Following a 50 ms pre-pulse to −20 mV, the membrane potential was returned to the holding potential (−100 mV) for a variable interval (the post-conditioning duration). This was followed by a test pulse to +10 mV (inset). Here the amplitude of the current generated by the test pulse (expressed as a percentage of current in the absence of the pre-pulse) is plotted against the post-conditioning duration. Note that the effect of the pre-pulse could be partially reversed by brief (5 ms) repolarization. The duration for half-reactivation was 12 ms (dotted lines).

resistance compensation. Current densities for different cells from the same animal were similar, irrespective of whether those cells came from the same or different pieces of perirhopalial tissue. For one animal, for instance, peak transient inward current densities ranged from 67 to 141 pA pF^{-1} (mean = 113·3 ± 15·5). There was considerable variation between animals, however, with densities overall ranging from 46 to 253 pA pF^{-1} (mean = 156·7 ± 15·5). The magnitude of this variation required than any comparative analysis of the effects of channel blockers etc. be done on cells from the same animal.

Ionic dependency

The amplitude of both components of inward current was reduced when the extracellular Na^+ concentration was reduced, implying that at least part of the inward current was carried by Na^+. This was confirmed in two ways. First, inward currents were recorded from a cell bathed in normal saline (390 mmol l^{-1} Na^+) (Fig. 4A). A pipette containing Na^+-free (tetramethylammonium, TMA^+, substituted) but otherwise normal saline was then positioned near the cell and a stream of Na^+-free saline directed at the cell. When the same voltage regime was applied to the cell while the Na^+-free stream was on, inward current was essentially abolished (Fig. 4A). The Na^+-free stream was then turned off and, after sufficient time to allow the Na^+-replete medium to diffuse back (approx. 2 min), a third family of voltage steps was applied. Currents elicited by this series were smaller than those in

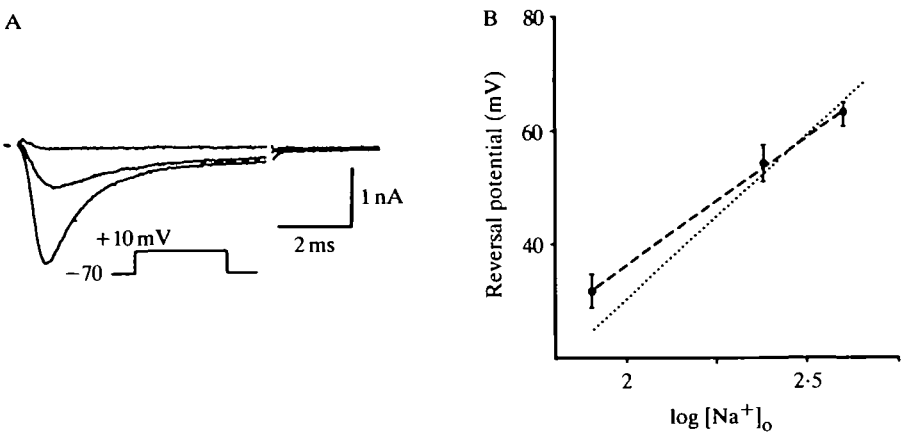

Fig. 4. Effect of $[Na^+]_o$ on inward current amplitude. (A) Inward current generated by the voltage step shown before (lower trace), during (upper trace) and after (middle trace) perfusion of the area around the cell with Na^+-free saline. Internal medium; Cs^+/TEA^+ patch solution with 2 mmol l^{-1} 4-aminopyridine (4-AP), 0 Na^+: external medium, saline or Na^+-free (TMA^+ substituted) saline with 7·5 mmol l^{-1} 4-AP. $[Ca^{2+}]$ 9·5 mmol l^{-1}. (B) Relationship between the extracellular Na^+ concentration and the reversal potential of the transient inward curent. Each point represents the mean ± S.E.M. for several trials from different cells. The dashed line is a least squares fit of the data and has a slope of 46 mV/decade. The dotted line represents the ideal line expected on the basis of the Nernst equation for a monovalent cation such as Na^+. Internal medium, as for Fig. 2, external medium, Ca^{2+}-free salines with different $[Na^+]$.

the first one but, nevertheless, inward current was once again present (Fig. 4A). The reduced amplitude of the inward current in the third trial probably reflects incomplete diffusion of the Na^+-replete medium, since the reversal potential of the currents recorded in the third trial was less positive than that in the first (not shown).

In the second series of experiments, inward currents were recorded in Ca^{2+}-free salines with different Na^+ concentrations. The intracellular (pipette) Na^+ concentration was kept constant at 30 mmol l^{-1}. The relationship between inward current reversal potential and extracellular Na^+ concentration (Fig. 4B) had a slope of 46 mV/decade. Although less than the value predicted by the Nernst equation, this value is sufficiently close to indicate that inward current in these cells is carried by Na^+.

As indicated earlier (Fig. 4A) inward current was abolished by the removal of extracellular Na^+. However, inward current was present in Na^+-free saline whose Ca^{2+} concentration was 10-fold higher than normal (Fig. 5A). Inward current in 95 mmol l^{-1} Ca^{2+} saline was a transient current. It was slower than that recorded in the presence of normal $[Na^+]$ and $[Ca^{2+}]$, taking 2–3 ms to reach peak amplitude. Decay from the peak current was also slower, with a time constant of 4–11 ms. Inward tail currents recorded at the end of the voltage step were obvious.

The current/voltage relationship of the current recorded in 95 mmol l^{-1} Ca^{2+} was very different from that of the current recorded in normal saline (Fig. 5B). Inward

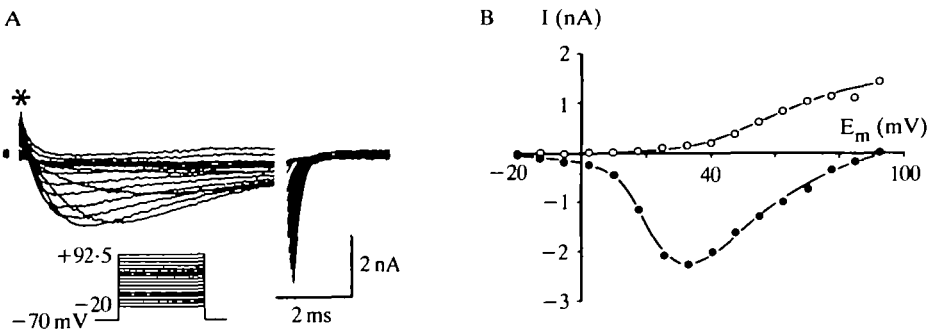

Fig. 5. (A) A family of inward currents recorded from a neurone bathed in Na^+-free (TEA^+ substituted) saline that contained 95 mmol l^{-1} Ca^{2+}. Voltage paradigm shown in the inset; voltage steps increased in 7·5-mV increments. Note that the waveform of the inward current is very different from that of the transient current described earlier (Fig. 2). These records also contain an early transient outward current (*). External medium, Na^+-free, 95 mmol l^{-1} Ca^{2+} saline; internal medium, Cs^+/TEA^+ patch solution with 30 mmol l^{-1} Na^+ and 2 mmol l^{-1} 4-aminopyridine. (B) Current/voltage relationships of the inward (●) and early transient outward (○) currents in A.

current activated at 0 to +10 mV, reached peak amplitude at +40 mV and reversed at around +90 mV. Inactivation was, once again, voltage-dependent. An H-infinity curve, produced in the same manner as before, provided a value of +15 mV for V_h.

The different waveforms and current/voltage relationships argue that the inward current recorded in Na^+-free, 95 mmol l^{-1} Ca^{2+} is different from the fast, transient inward current described earlier. This is given added support by the observation that currents recorded in Na^+-free, 95 mmol l^{-1} Ca^{2+} saline with pipettes that contained 30 mmol l^{-1} Na^+ included a fast, transient, outward current at the onset of voltage steps to potentials greater than +15 mV. These transient outward currents had the appearance and time course of the fast, transient current recorded in normal saline at potentials more positive than its reversal potential. While it is conceivable that the transient outward currents may represent unblocked outward (K^+) current, the fact that they were reduced or absent if Na^+ were omitted from the pipette suggests that they indeed represent the efflux of Na^+ through the normal Na^+ channel.

Currents recorded in Cl^--free saline with Cl^--free patch solution were very similar to those recorded with Cl^- present except that their current/voltage relationships were shifted some 10–20 mV to the left.

Pharmacology

Both components of inward current could be recorded in the presence of 0·1 mmol l^{-1} TTX with no apparent change in waveform. To determine if a portion of the total inward current had been blocked by TTX at this concentration, inward currents were recorded from different cells from the same animal, in the presence and absence of 0·1 mmol l^{-1} TTX. With one animal, the mean current density was 113·3 ± 15·5 pA pF^{-1} in the absence of TTX and 130·6 ± 28·3 pA pF^{-1} in the

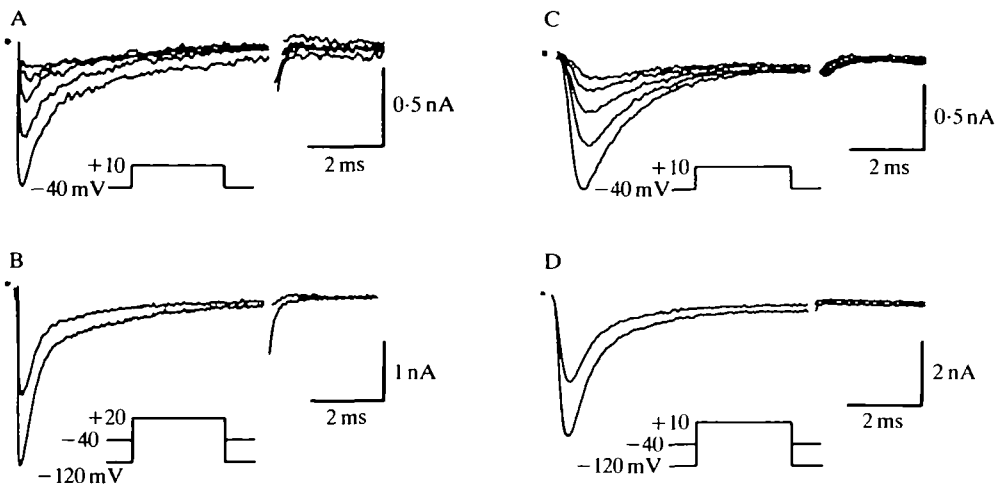

Fig. 6. (A) Effect of repetitive stimulation on inward currents recorded in the presence of 1 mmol l^{-1} lidocaine. Currents generated by the first, fourth, eighth, twelfth and sixteenth of a series of identical voltage steps (inset) are superimposed. (B) Superimposed currents generated by step depolarizations to +20 mV from holding potentials of −120 mV and −40 mV. (C) Inward currents recorded in the same manner as those in A, but in the presence of 20 μmol l^{-1} W7. (D) Currents recorded in the presence of 20 μmol l^{-1} W7, following depolarizations to +10 mV from holding potentials of −120 and −40 mV. External media, saline with 7·5 mmol l^{-1} 4-aminopyridine (4-AP) and lidocaine or W7; internal media, Cs^+/TEA^+ patch solution with 2 mmol l^{-1} 4-AP and 30 mmol l^{-1} Na^+.

presence of 0·1 mmol l^{-1} TTX, indicating that no current had been blocked by TTX. Tetrodotoxin was not used in any of the following pharmacological experiments.

Saxitoxin (10 μmol l^{-1}) and GIIIA (1 μmol l^{-1}), a cone toxin which blocks Na^+ channels in muscle but not nerve (Moczydlowski, Olivera, Gray & Strichartz, 1986), were both completely ineffective as were batrachotoxin (2 μmol l^{-1}), veratridine (100 μmol l^{-1}), *Leiurus* venom (1 μmol l^{-1}) and sea anemone, *Stichodactyla* (=*Stoichactus*), toxin (1 μmol l^{-1}), all agents which block or remove Na^+ channel inactivation (Hille, 1984).

Inward current could, however, be blocked by lidocaine, verapamil, Cd^{2+} and W7. 1 mmol l^{-1} lidocaine reduced the amplitude of the transient inward current by 77 %. At a lower dose (0·25 mmol l^{-1}), the reduction was only 42 %. The effect of lidocaine was membrane potential- and use-dependent (Fig. 6A,B); with repetitive stimulation, the current amplitude decreased continuously (Fig. 6A) and currents generated from depolarized holding potentials (−40 mV) were far less than those obtained at very negative holding potentials (−120 mV) (Fig. 6B). These effects of lidocaine are consistent with its known action on other excitable cells (for a review, see Hille, 1984).

The steady-state inward current was apparently unaffected by lidocaine at this concentration and because it was largely uncontaminated by the transient current,

its waveform and voltage-dependency could be distinguished more clearly. The lidocaine-insensitive steady-state current was small, activated at −5 mV and typically reversed some 10–20 mV more positive than the residual transient inward current. Because some transient inward current invariably remained under these conditions the time constant of activation of the steady-state current could not be determined. However, peak amplitude was reached 2–3 ms after the onset of the voltage step. The appearance and properties of this current, to the extent examined, were very similar to those of the slow, inward current recorded in Na^+-free, high-Ca^{2+} saline (Fig. 5A).

The effect of W7 was very similar to that of lidocaine. At low concentration ($20\,\mu mol\,l^{-1}$), W7 produced a reversible, use- and potential-dependent block of the transient inward current (Fig. 6C,D). At higher concentrations ($100\,\mu mol\,l^{-1}$), inward current was blocked completely although, interestingly, depolarizing steps to potentials more positive than +40 mV produced a small, but obvious, transient outward current at the onset of the voltage step. The waveform of this current was very similar to that of the normal, inward, transient current after reversal suggesting that W7 was blocking the influx of Na^+ completely, but not its efflux. $100\,\mu mol\,l^{-1}$ W7 did not block the steady-state inward current completely and, with some cells, currents very similar to those recorded in $95\,mmol\,l^{-1}$ Ca^{2+}, $0\,mmol\,l^{-1}$ Na^+ were recorded in the presence of $100\,\mu mol\,l^{-1}$ W7.

The effect of verapamil differed from that of lidocaine and W7 inasmuch as both components of inward current were blocked by verapamil. $2\times10^{-6}\,mol\,l^{-1}$ verapamil blocked 30 % of the transient inward current and at $0{\cdot}1\,mmol\,l^{-1}$ 90 % was blocked. There was no obvious change in the I/V relationships of the residual currents. Inward current was also blocked by extracellular Cd^{2+}. Once again, the effect was dose-dependent; with one cell, $5\,mmol\,l^{-1}$ Cd^{2+} produced a 74·7 % reduction in transient inward current, with another cell, $2{\cdot}5\,mmol\,l^{-1}$ Cd^{2+} produced a 59 % reduction. Although an obvious reduction in current density was observed, there was no change in the waveform or voltage-dependency of the residual inward current. Both components of inward current were equally affected.

DISCUSSION

The major inward current in the motor nerve net neurones of *Cyanea* is a fast, transient current whose kinetics are very similar to those of Na^+ currents in more classical preparations. That Na^+ is indeed the charge carrier is evident from the observations that the inward current was abolished by removal of extracellular Na^+ (Fig. 4A) and that the reversal potential was dependent on the extracellular Na^+ concentration (Fig. 4B). The slope of the relationship between the reversal potential and the Na^+ concentration (Fig. 4B) (46 mV decade) is lower than that predicted by the Nernst equation, but the discrepancy can probably be explained by the fact that these data were obtained using Na^+ concentration rather than Na^+ activity, which

would have given a more realistic measure of available Na^+, particularly at the higher concentrations.

The fact that inward current existed in Na^+-free, 95 mmol l^{-1} Ca^{2+} saline (Fig. 5) might be interpreted as indicating that the inward current channels are normally selective for Na^+ but, in its absence, will permit a Ca^{2+} flux. This possibility can be excluded, however, for several reasons. The time course of the currents in Na^+-free, high-Ca^{2+} saline was significantly slower than that of the normal inward current (Fig. 5) and the voltage-dependencies of these currents were shifted in the positive direction. These findings strongly suggest that there are two distinct types of inward current, but they are insufficient justification on their own, since the different kinetics could be because Ca^{2+} is flowing, rather than Na^+, and the different voltage-dependency could be attributed to a modification of the normal voltage-dependency by the elevated Ca^{2+} concentration (Frankenhaeuser & Hodgkin, 1957). However, during these experiments with Na^+-free, high-Ca^{2+} saline, a fast, transient, outward-going current, with the time course of the normal Na^+ current after its reversal, occurred at the onset of voltage steps to potentials more positive than +15 mV (Fig. 5), but only if Na^+ were present in the patch solution. The kinetics of this current and its requirement for intracellular Na^+ suggest then that this current represents the outward movement of Na^+ through the normal Na^+ channels. Since those channels inactivate and would then be unavailable to other ions, the inward current recorded in this medium (Fig. 5) must be using a second population of ion channels. Thus, there appear to be two classes of inward current channels in these cells; fast, transient channels, selective for Na^+ over Ca^{2+}, and slower, transient, presumably Ca^{2+}-selective channels. The properties of the latter channels will be discussed later.

The Na^+ current in these cells differs from Na^+ currents in other excitable cells in its voltage-dependency and TTX-sensitivity. The voltage-dependencies of the Na^+ current were shifted in the positive direction. The inward current activated between −20 and −10 mV and V_h, the voltage corresponding to one-half inactivation (Fig. 3A), was −15 mV. In most other cell types, Na^+ currents are activated by depolarizations to −50 to −60 mV and V_h is usually more negative; −75 mV for frog myelinated nerve fibres, for instance (Hille, 1984). The reason for the unusually high threshold is unclear. It may be an adaptation to the bidirectionality of the synapses made by these neurones; a means of preventing the 'echo' synaptic potentials (Anderson, 1985) from triggering other action potentials, thereby releasing additional transmitter from the presynaptic terminals and making the synapses tonic. This is unlikely, however, since inward current would be inactivated following the first action potential and, as has been shown (Fig. 3B), these cells must be repolarized for 50 ms for complete recovery from inactivation.

Alternatively, the positive shift in the voltage-dependency of this current may be a normal feature of 'primitive' ion channels. Inward currents in other coelenterate cells tend to activate at less negative potentials than their equivalents in other organisms (Anderson & McKay, 1987; Dubas & Anderson, 1986; Dunlap, Takeda & Brehm, 1987), as do inward currents in protozoans (Naitoh, 1982; Oertel, Schein & Kung,

1977). The reason is unclear and additional voltage-clamp studies of inward currents will be required to determine how widespread this phenomenon is in these animals.

The other remarkable feature of the Na^+ current in these cells is that it was completely insensitive to TTX (0·1 mmol l^{-1}) and STX (10 mmol l^{-1}). This marked TTX-insensitivity is not unusual in cnidarians where TTX-sensitive action potentials have yet to be described (Anderson & Schwab, 1982). The other notable group with TTX-insensitive Na^+ currents are leeches. There, Na^+-dependent action potentials in some cells are TTX-sensitive, others are not (Kleinhaus & Prichard, 1976; Yang, Johansen & Kleinhaus, 1984). Furthermore, in some neurones there are two distinct Na^+ currents with differing kinetics and TTX (STX) sensitivities (Kleinhaus & Johansen, 1986). However, TTX is normally a highly specific and extremely potent blocker of Na^+ channels in electrically excitable cells. With most cells nanomolar concentrations of TTX are sufficient to block Na^+ currents but in denervated muscle and developing and cultured neurones micromolar concentrations are sometimes required (Harris & Marshall, 1973; Kidokoro *et al.* 1975; Pappone, 1980). In the latter cases, the currents are sometimes erroneously referred to as TTX-insensitive currents; TTX-resistant would be a better descriptor. In any case, the specificity of TTX has meant that TTX-sensitivity alone is usually sufficient evidence for confirming the Na^+-dependency of an action potential. However, the converse argument, that TTX-insensitivity (as opposed to TTX-resistance) means that a spike is not Na^+-dependent, does not necessarily apply. TTX-sensitivity is not a functional requirement for a Na^+ channel, merely a coincidental one. For instance, a 2-min exposure to trimethyoxonium ions makes Na^+ channels in frog nerve insensitive to TTX (presumably by methylating one or more exposed groups) without affecting the Na^+ currents unduly (Hille, 1984; Reed & Raftery, 1976; Spalding, 1980). In addition, Na^+ channels in animals which produce TTX for defence, and those in animals frequently exposed to STX, are very resistant to the toxins (Kao & Fuhrman, 1967; Kidokoro, Grinnell & Eaton, 1974; Twarog, Hidaka & Yamaguchi, 1972). Thus, the TTX-insensitivity of the fast, transient, inward current in *Cyanea* neurones may simply result from a minor, structural modification of the otherwise normal Na^+ channel. However, the fact that the Na^+ currents were also totally unaffected by BTX, veratridine, scorpion venom and sea anemone toxin, agents which normally interfere with Na^+ inactivation, suggests that these Na^+ channels may be quite different from those in higher animals.

This suggestion is supported by the observation that the Na^+ current was blocked in a dose-dependent manner by Cd^{2+} and verapamil, agents usually considered to be Ca^{2+} channel blockers. However, the picture here is somewhat fuzzy since Ca^{2+} channel blockers are not as specific as TTX, particularly when used at the higher concentrations usually required for invertebrate tissues. For instance, Cd^{2+} will also block some K^+ channels (Matteson & Deutsch, 1984), Na^+ channels in mature cells (Bowers, 1985; Difrancesco, Ferroni, Visentin & Zaza, 1985), and TTX-resistant Na^+ channels in embryonic (Spitzer, 1979) and cultured (Fukuda & Kameyama 1980; Yoshida, Matsuda & Samejima, 1978) neurones. Therefore, although the Cd^{2+} concentrations used here were consistent with those used upon other invertebrate

tissues (Adams *et al.* 1980), they are higher than those used to block Ca^{2+} currents in vertebrate preparations, raising the possibility that the action of Cd^{2+} here is non-specific. Similarly, while verapamil is a Ca^{2+} channel blocker, high concentrations of verapamil (0·1–1 mmol l^{-1}) will block Na^+ currents in *Aplysia* neurones (Adams & Gage, 1979). However, with *Cyanea* neurones, a significant block of inward current occurred with micromolar verapamil, suggesting that the effect here may be more specific. The third Ca^{2+} channel antagonist used here, W7, blocked the transient Na^+ current in a voltage- and use-dependent manner (Fig. 6C,D). The fact that a transient outward current appeared during positive voltage steps suggests that W7 occluded the channel from the outside, thereby preventing Na^+ influx but not its efflux.

It should be stressed that the sensitivity of the fast, transient, inward current to Ca^{2+} channel blockers and its complete insensitivity to classical Na^+ channel blockers does not mean that this is a Ca^{2+} current; the channels are selective for Na^+ (Fig. 4). These findings indicate that although this inward current has the kinetics and ionic dependencies of a Na^+ current, its pharmacology is more reminiscent of that of Ca^{2+} currents.

Interestingly, it has been proposed (Hille, 1984) that Na^+ channels may have evolved from Ca^+ channels to permit axons to fire at high frequencies without compromising them by loading them with Ca^{2+}. If so, this development presumably took place in the Cnidaria since it is the first phylum to possess neurones and to produce Na^+-dependent action potentials. Neurones in the more advanced platyhelminths are sensitive to TTX (Koopowitz & Keenan, 1982). If this suggestion is correct, the contradiction between the physiology and pharmacology of the Na^+ current in *Cyanea* cells may reflect their position in this evolutionary process.

The motor nerve net neurones are connected by bidirectional, excitatory chemical synapses. The threshold for transmitter release at these synapses is unusually high (Anderson, 1985), and this may reflect the presence of a high threshold Ca^{2+} current. The slower inward current recorded in Na^+-free, 95 mmol l^{-1} Ca^{2+} saline (Fig. 5) or normal saline with lidocaine or W7, may be this current. This current can be distinguished from the fast, transient Na^+ current on the basis of the different waveform, voltage-dependence and other factors discussed earlier. Its requirements for external Ca^{2+}, but not Na^+, its insensitivity to lidocaine, and its very depolarized activation potential are consistent with the known requirements for transmission at these synapses (Anderson, 1985). This presumed Ca^{2+} current is a transient current. Its inactivation is voltage-dependent ($V_h = +15$ mV) although it is possible that there may also be Ca^{2+}-dependent inactivation (Eckert & Chad, 1984). To date, three types of Ca^{2+} current (I_T, I_L and I_N) have been described (Nowycky, Fox & Tsien, 1985) but whether the Ca^{2+} current in these cells falls into any of these categories remains to be seen.

The waveforms of this Ca^{2+} current and the lidocaine- and W7-insensitive currents are very similar, although more work is needed to confirm this. It is interesting to note, however, that the lidocaine-insensitive inward current activated at a membrane potential of −5 mV. Since those currents were recorded in normal

Ca^{2+} concentrations, this activation potential is comparable to that of the presumed Ca^{2+} current where the elevated $[Ca^{2+}]_o$ would have shifted the gating properties in the positive direction (Frankenhaeuser & Hodgkin, 1957). If the W7-insensitive current is a Ca^{2+} current, the fact that it is relatively insensitive to W7 but blocked by verapamil suggests that the Ca^{2+} current in these cells is quite different from those in *Paramecium* which are blocked by W7 (Hennessey & Kung, 1984) but not blocked by TTX or the methoxy-derivative of verapamil, D600 (Eckert & Brehm, 1979).

The identity of the current that remains after inactivation of the transient Na^+ current is unclear. Its reversal potential was usually close to that of the transient inward current (Fig. 2B). This suggests that it, too, is a Na^+ current, but its function is unclear, particularly since its relative amplitude varied considerably from cell to cell. One possibility is that the steady-state current may be an artefact introduced by the use of patch pipettes. Na^+ currents in squid axons usually inactivate totally, but in internally perfused axons a significant steady-state component remains (Bezanilla & Armstrong, 1977; Chandler & Meves, 1970*a*,*b*). Alternatively, the steady-state component may be the presumed Ca^{2+} current. Indeed, under the conditions employed here, a portion of the steady-state current must have been carried by Ca^{2+} since Ca^{2+} was present and its reversal potential may have been lowered by residual outward current which would have been maximally active at that time. If the steady-state inward current is the Ca^{2+} current, the variations in current amplitude could reflect the synaptic geometry of the cells; in this nerve net, synapses are formed apparently randomly, on all parts of the cell, by all parts of the cell. Thus, those records that contained relatively large amounts of steady-state current may have come from somata that contained presynaptic terminals; those without any steady-state current may have come from areas which lacked synapses. The properties and ionic dependencies of the steady-state inward current may become evident when the Ca^{2+} current has been examined in more detail.

In conclusion, voltage-clamp studies of ionic currents in coelenterate tissues (Anderson & McKay, 1987; Barrish, 1983; Dubas & Anderson, 1986; Dunlap *et al.* 1987; Hagiwara, Yoshida & Yoshii, 1981) indicate that these currents are similar to those of higher animals. To date, however, fast Na^+ currents have never been examined in these animals. The results presented here indicate that although the kinetics of the Na^+ current in the motor nerve net neurones of *Cyanea* are similar to those of Na^+ currents in other animals, their pharmacology is quite different, being more reminiscent of that of Ca^{2+} currents than Na^+ currents. This finding may indicate that the Na^+ channels in these cells are intermediates in the evolution of the Na^+ channels of higher organisms, as discussed, or indicate that the Na^+ channels of cnidarian neurones are very different from those of higher animals yet produce currents with similar kinetics. More work is obviously required to resolve these questions.

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