

Na⁺, K⁺ AND Ca²⁺ CURRENTS IN IDENTIFIED LEECH NEURONES IN CULTURE

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Summary

1. Na⁺, K⁺ and Ca²⁺ currents have been measured by voltage-clamp in Retzius (R), anterior pagoda (AP) and sensory (pressure, touch and nociceptive) cells dissected from the central nervous system (CNS) of the leech. These cells maintain their distinctive membrane properties and action potential configurations in culture. Currents carried by the individual ions were analysed by the use of channel blockers and by their kinetics. Since the cells are isopotential they can be voltage-clamped effectively.

2. Depolarization, as expected, gave rise to an early inward Na⁺ current followed by a delayed outward K⁺ current. In Na⁺-free medium containing tetraethylammonium (TEA⁺), and in the presence of 4-aminopyridine (4-AP), inward Ca²⁺ currents were revealed that inactivated slowly and were blocked by Cd²⁺ and Mn²⁺.

3. Na⁺ and Ca²⁺ currents were similar in their characteristics in R, AP and sensory neurones. In contrast, K⁺ currents showed marked differences. Three principal K⁺ currents were identified. These differed in their time courses of activation and inactivation and in their responses to Ca²⁺ channel blockers.

4. K⁺ currents of the A-type (I_A) activated and inactivated rapidly, were not affected by Ca²⁺ channel blockers and were eliminated by steady-state inactivation at holding potentials of −30 mV. A-type K⁺ currents were found in AP cells and as a minor component of the outward current in R cells. A Ca²⁺-activated K⁺ current (I_C), that inactivated more slowly and was reduced by Ca²⁺ channel blockers, constituted the major outward current in R cells. The third K⁺ current resembled the delayed rectifier currents (I_{K1} and I_{K2}) of squid axons with slow activation and inactivation kinetics. Such currents were found in R cells and in the sensory neurones (T, P and N).

5. The principal differences in membrane properties of identified leech neurones can be explained in terms of the numbers of Na⁺ channels and the distinctive kinetics of K⁺ channels in each type of cell.

Introduction

Detailed information is available about the properties of identified leech

Key words: leech, voltage-clamp, K⁺ currents, cultured neurones.

neurones. For many nerve cells of known function, the morphology, the transmitter chemistry and the synaptic connections have been studied within the CNS; from cell type to cell type there are striking differences in action potential configuration, delayed rectification, long-lasting Ca^{2+} -activated K^+ conductances and other variables that influence signalling. Each cell has an ensemble of properties by which it can be unambiguously recognized (Nicholls, 1987). Moreover, after an identified neurone has been removed from the ganglion and placed in culture, it retains these properties. For example, sensory cells continue to give highly distinctive action potentials for days or weeks in culture.

At the same time, surprisingly little is known about how the various identified nerve cells differ with respect to the ion channels they exhibit. From experiments made on neurones within ganglia, it is known that action potentials in leech neurones depend on Na^+ , are unaffected by moderate concentrations of tetrodotoxin (Nicholls & Kuffler, 1964; Kleinhaus & Prichard, 1983; Beleslin, 1985) and are prolonged by TEA^+ and 4-aminopyridine (Kleinhaus, 1976; Kleinhaus & Prichard, 1975; Johansen & Kleinhaus, 1985, 1986). However, numerous technical difficulties arise if one wishes to compare with voltage-clamp the ionic currents that give rise to the membrane properties in different types of cells. These include: (1) the large sizes of the cells which makes conventional whole-cell patch-clamp difficult to use; (2) the high resistances of the microelectrodes necessary for penetrating leech neurones when using two-electrode voltage-clamp; (3) the complex arborizations of the cells *in situ* which prevent adequate space clamping; and (4) the ineffectiveness of tetrodotoxin.

In this paper we describe voltage-dependent Na^+ , K^+ and Ca^{2+} currents in Retzius (R), anterior pagoda (AP) and sensory [pressure (P), nociceptive (N) and touch (T)] cells in culture. A particular advantage of the cells in culture is that they can be effectively voltage-clamped because they are isopotential spheres when plated on polylysine, a substrate that does not promote process outgrowth (Chiquet & Acklin, 1986; Dietzel *et al.* 1986; Ross *et al.* 1987). Emphasis has been placed on R and AP cells, which display markedly different K^+ currents, and on P sensory cells with which R cells form chemical synapses in culture (Fuchs *et al.* 1982). Divalent cation currents are described in more detail for R cells elsewhere (R. J. Bookman & Y. Liu, in preparation). These experiments constitute a necessary first step in the analysis of currents responsible for generating the distinctive action potentials of identified cells. Moreover, information about manipulations required for blockage of Na^+ and K^+ currents will make it possible to correlate inward Ca^{2+} currents with transmitter release at synapses formed in culture by R and P cells.

Materials and methods

Removal and culture of leech neurones

The methods for removing and culturing leech neurones have already been described (Fuchs *et al.* 1981; Dietzel *et al.* 1986). R, AP, T, N and P cells identified

by their sizes and positions were removed from desheathed ganglia by lassoing them with nylon monofilament loops or by suction after treatment for 1 h at room temperature with collagenase and dispase (2 mg ml^{-1} ; Boehringer-Mannheim Corporation). The diameters of these neurones were, in order from largest to smallest, R ($80\text{--}100 \mu\text{m}$) > AP > P > N > T ($50 \mu\text{m}$). Individual cells were placed in Leibowitz-15 (L-15, Gibco) medium supplemented with 0.1 mg ml^{-1} gentamycin, 6 mg ml^{-1} glucose and 2 % foetal calf serum and plated onto polylysine-coated microwells (Falcon 3034F, type 60) for 1–14 days at 20°C .

Electrical recordings

Two-electrode voltage-clamp (Almost Perfect Electronics, Basel, Switzerland) was used to measure the voltage-dependent ionic currents in each cell type (see Fig. 1A). Approximately 75 % of the cells penetrated yielded useful results. All measurements were made at room temperature ($22\text{--}25^\circ\text{C}$). Cells were observed in an inverted microscope. Electrodes were pulled from Haer 30–30–0 thin-walled microelectrode glass and filled with 4 mol l^{-1} potassium acetate. $10\text{--}20 \text{ M}\Omega$ electrodes were used for passing current and $10\text{--}30 \text{ M}\Omega$ electrodes for measuring voltage. The voltage was recorded differentially (see Fig. 1A) and was connected to the feedback circuit of the voltage-clamp. A large, grounded aluminium shield was interposed between the electrodes to reduce capacitive coupling between them. Owing to the high resistance of the electrodes that are needed to penetrate leech neurones, a number of special precautions were necessary to ensure adequate clamping and short time constants. The current signal from the voltage-clamp was fed through a high-frequency amplifying circuit (Almost Perfect Electronics, Basel, Switzerland) to compensate for the time constants of the two electrodes and was connected to the current input of a second preamplifier. This, in turn, was connected to a high-voltage, constant-current head stage ($\pm 100 \text{ V}$) which was used to pass current through the current-passing microelectrode. Current and voltage signals were stored on magnetic tape and transferred to a computer (MINC/DECLAB-23) for analysis as described below.

Solutions

For measuring the total current evoked by depolarizing voltage steps, the external solution was standard leech Ringer's fluid to which *N*-methyl-D-glucamine chloride was added to bring the osmolality to between 350 and 370 mmol kg^{-1} . This corresponds to the tonicity of the L-15-enriched solutions in which the neurones were cultured. The solutions used for measuring individual currents are listed in Table 1.

A gravity-fed perfusion system was used to exchange bathing solutions. The solution was fed into the microwell through a pipette and the height of the solution reservoir as well as the diameter of the tip of the pipette were adjusted to regulate the flow rate. Typically, a complete change of solution, as judged by changes in ionic currents, was achieved in 2–3 min.

Table 1. *Solutions*

| | NaCl (mmol l ⁻¹) | KCl (mmol l ⁻¹) | Divalent cation (mmol l ⁻¹) | TEA-Cl* (mmol l ⁻¹) | 4-AP† (mmol l ⁻¹) |
|--|---------------------------------|--------------------------------|--|------------------------------------|----------------------------------|
| 1 Normal Na ⁺ | 125 | 4 | 2 (Ca ²⁺) | | • |
| 2 1/2-normal Na ⁺ I | 62.5 | 4 | 2 (Ca ²⁺) | | |
| 3 1/2-normal Na ⁺ II | 62.5 | 4 | 2 (Mn ²⁺) | 100 | |
| 4 Low-Na ⁺ | 10 | 4 | 2 (Ca ²⁺) | | |
| 5 Low-Na ⁺ , TEA ⁺ | 10 | 4 | 2 (Ca ²⁺) | 25-100 | |
| 6 Low-Na ⁺ , 4-AP | 10 | 4 | 2 (Ca ²⁺) | | 5-10 |
| 7 Low-Na ⁺ , Cd ²⁺ | 10 | 4 | 2 (Ca ²⁺) 0.1 (Cd ²⁺) | | |
| 8 High-K ⁺ I | 10 | 23 | 2 (Ca ²⁺) | | |
| 9 High-K ⁺ II | 10 | 23 | 2 (Ca ²⁺) | 100 | |
| 10 High-K ⁺ III | 10 | 40 | 2 (Ca ²⁺) | | |
| 11 High-Ca ²⁺ I | 10 | 4 | 3 (Ca ²⁺) | | |
| 12 High-Ca ²⁺ II | 10 | 4 | 10 (Ca ²⁺) | 100 | |
| 13 High-Ca ²⁺ III | 10 | 4 | 10 (Ca ²⁺) | 100 | 5 |
| 14 Ca ²⁺ -free, Mn ²⁺ I | 10 | 4 | 3 (Mn ²⁺) | | |
| 15 Ca ²⁺ -free, Mn ²⁺ II | 10 | 4 | 10 (Mn ²⁺) | 100 | |

Solutions were buffered to pH 7.4 with 10 mmol l⁻¹ Tris maleate. Osmolality was adjusted to between 350 and 370 mmol kg⁻¹ with *N*-methyl-D-glucamine chloride (NMG-Cl).

* TEA-Cl, tetraethylammonium chloride.

† 4-AP, 4-aminopyridine.

Data analysis

After transfer to a computer, current records produced by depolarizing voltage pulses had their capacitative transients and leak currents subtracted. The current records used for subtraction were produced either by a hyperpolarizing pulse (p) of equal magnitude to the depolarizing pulse or by three hyperpolarizing pulses (p/3) each one-third the magnitude of the depolarizing pulse. Holding currents were then subtracted to give a zero-baseline before further analysis. The activation time ($t_{1/2}$, time to one-half peak amplitude) and inactivation time constant (τ_h) of the current were determined. In some experiments, a family of current and voltage traces was used to generate peak current-voltage curves. In others, the effect of a blocking agent was monitored by measuring against time the current produced by pulses to a particular voltage.

Statistical comparison was made using Student's *t*-test, and differences were considered significant at $P < 0.05$.

Results

Separation of Na⁺, K⁺ and Ca²⁺ currents in isolated cells

In saline containing normal or half-normal [Na⁺] (solutions 1 and 2, Table 1)

step depolarizations of the membrane potential (from -50 mV to between -30 and $+10$ mV) produced a rapid inward current followed by a delayed outward current.

Hyperpolarization of the membrane produced only capacitative artefacts at the onset and offset of the pulse and a small, sustained leakage current during the pulse. Examples of these currents in an R cell are shown in Fig. 1B,C. The following questions have been examined. (1) Which ions carry these currents? (2) How can each current be separated from the others? (3) How do the separate ionic currents differ in the various cell types?

Initial inward Na^+ currents

In leech neurones, although external Na^+ is necessary for action potentials, moderate concentrations of tetrodotoxin or saxitoxin have no effect (Beleslin, 1985; Johansen & Kleinhaus, 1987). To determine whether Na^+ was responsible for the initial inward current, the external Na^+ concentration was lowered from 125 to 10 mmol l^{-1} while applying depolarizing steps to -5 mV (solutions 1 and 4, Table 1). In all five types of cell (R, AP, P, N and T) as the concentration of Na^+ decreased during the exchange of solutions the inward current decreased and eventually reversed. After replacing normal- Na^+ saline the inward current reappeared (Fig. 2).

Activation kinetics for the Na^+ current were too fast to be measured by conventional two-electrode voltage-clamp, owing to the large capacitance artefacts (see Materials and methods). They are described elsewhere (R. J. Bookman, J. G. Nicholls, H. Reuter & W. B. Adams, in preparation). Inactivation kinetics could, however, be analysed after reduction of Na^+ to 50% of normal concentration (which allowed for better control of membrane voltage during the peak Na^+ current), while Mn^{2+} and TEA^+ blocked Ca^{2+} and K^+ currents (solution 3, Table 1; Fig. 3A). There were no significant differences between inactivation time constants of Na^+ currents in the different types of cultured cells. Fig. 3B shows time constants of inactivation in P and R cells with graded voltage steps from a holding potential of -50 mV. For voltage steps from -11 to -1 mV, Na^+ currents were sufficiently large and slow to be measured accurately.

In contrast to the similarity between Na^+ inactivation kinetics in different cell types, the maximum peak Na^+ current and the current density showed marked differences. The membrane surface areas of R and P cells were estimated from measurements of membrane capacitance derived from input resistance and time constant. For R cells the values were: 2.0 ± 0.2 nF and 16 ± 4 M Ω (mean and s.e., $N=7$); for P cells the corresponding values were: 0.9 ± 0.4 nF and 15 ± 4 M Ω (mean and s.e., $N=7$). The mean maximum peak Na^+ current density calculated in this way for R cells was 11 ± 2 $\mu\text{A cm}^{-2}$ (mean and s.e., seven cells) and for P cells was 53 ± 11 $\mu\text{A cm}^{-2}$ (mean and s.e., seven cells). The difference between the means was highly significant ($P < 0.005$). Not surprisingly, Na^+ current density was greatest in cells that produced the largest action potentials, and was smallest in AP cells.

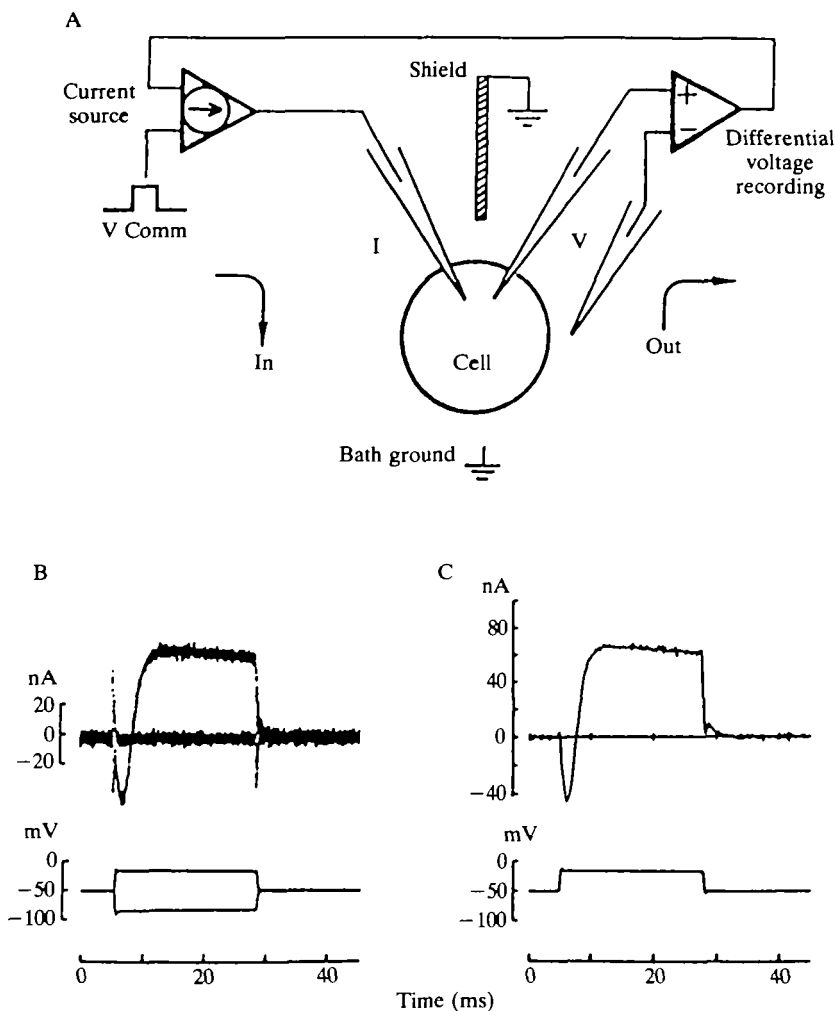


Fig. 1. Measurement of voltage-dependent ionic currents in a Retzius (R) cell. (A) Electrode configuration, showing two electrodes in a cell and a grounded shield between the electrodes. In and Out represent the perfusion system. V Comm, voltage command. (B) Current (top traces) and voltage (bottom traces) recordings from a voltage-clamped R cell in normal Na^+ medium (solution 1, Table 1). Hyperpolarization of the membrane potential from -50 to -83 mV produced a small leakage current. Depolarization of the membrane potential from -50 to -17 mV resulted in an early inward (–) current followed by a delayed outward (+) current. Capacitative artefacts occurred in the current records during abrupt changes in membrane voltage. (C) Leak subtracted and averaged current records ($N=9$) (top trace) and depolarizing voltage step (bottom trace) shown in B. The delayed outward current inactivated with time and upon membrane repolarization outward tail currents were detectable.

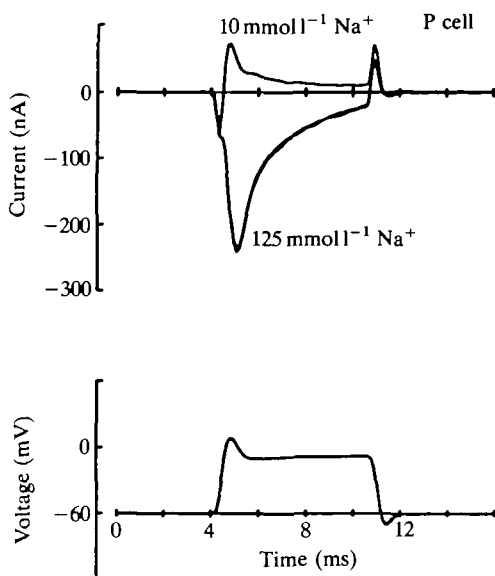


Fig. 2. Na⁺-dependence of early inward current in a P cell. Na⁺ currents were recorded in normal external (125 mmol l⁻¹) Na⁺ medium and low (10 mmol l⁻¹) Na⁺ medium (solutions 1 and 4, Table 1). Na⁺ currents were large and inward in normal Na⁺, small and outward in low external Na⁺ and reappeared in normal Na⁺ medium. Each trace is leak subtracted and averaged ($N = 10$).

Inward divalent cation currents

Neurones bathed in low-Na⁺, TEA⁺ medium containing 10 mmol l⁻¹ Ca²⁺, Ba²⁺ or Sr²⁺ exhibited inward currents upon step depolarization to between -5 and +30 mV. Examples of inward Ca²⁺ currents in AP, R, P and N cells are shown in Figs 4 and 6A (solutions 5 and 12, Table 1). In all experiments, these inward currents activated and inactivated more slowly than Na⁺ currents and were unaffected by changes in external [Na⁺]. Cd²⁺ and Mn²⁺ blocked the divalent cation current reversibly. A slow decline in inward Ca²⁺ current was observed in R, N and P cells, but not in AP cells (Figs 4, 6A). Whether this represents inactivation of Ca²⁺ channels or the result of slow, unblocked outward currents or a combination of both would not be resolved by our techniques. Further analysis of divalent cation currents in R cells is presented elsewhere (R. J. Bookman & Y. Liu, in preparation).

Delayed outward K⁺ current

Experiments were performed which demonstrated that the delayed outward current shown in Fig. 1 was carried by K⁺. The external K⁺ concentration was increased to change E_K , the equilibrium potential. In leech neurones it has been shown that E_K changes from -85 to -27 mV when the external K⁺ concentration is increased from 4 to 40 mmol l⁻¹ (Nicholls & Kuffler, 1964). In 40 mmol l⁻¹ K⁺ solution, during a step depolarization from -50 to +10 mV, the driving force for

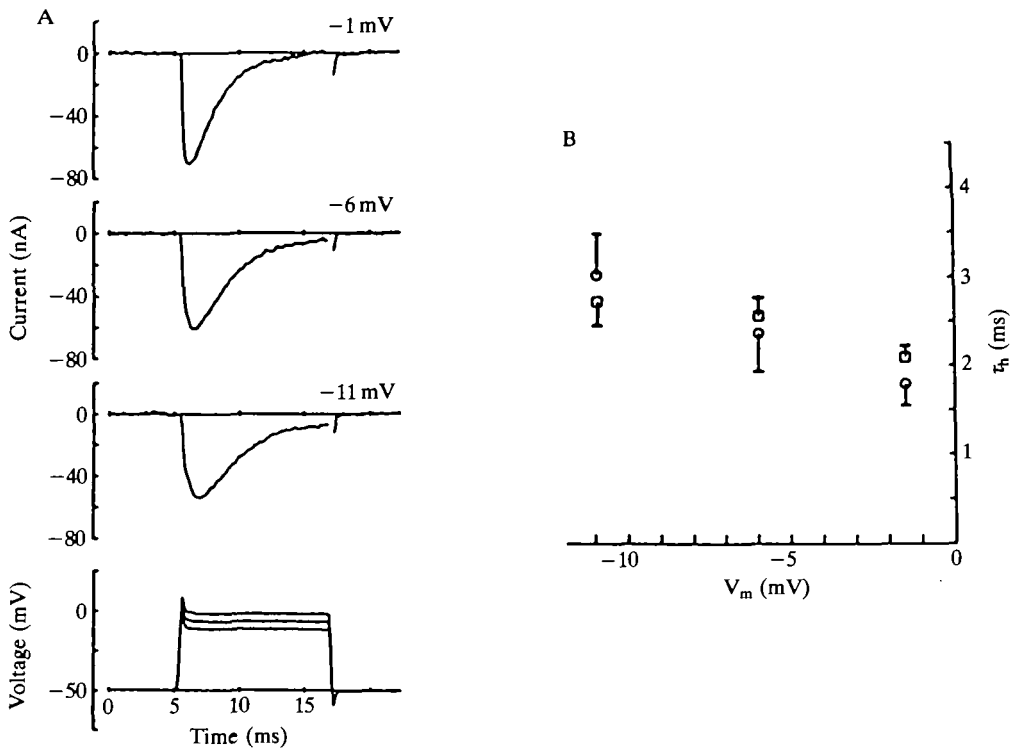


Fig. 3. (A) Na^+ currents recorded in a P cell bathed in 50% normal Na^+ concentration; K^+ and Ca^{2+} currents were blocked by TEA^+ and Mn^{2+} (solution 3, Table 1). The record at the bottom is of membrane voltage and indicates a holding potential of -50 mV. The magnitude of each voltage step is shown to the right of its corresponding current record. Each trace is leak-subtracted and averaged ($N = 9$). Stimulus artefacts are not shown. The settling time of the clamp was approximately 1 ms. (B) Inactivation time constants for Na^+ currents measured at three different depolarizing voltages in R (circles) and in P (squares) cells. The bars represent standard errors of the means ($N = 7$ for R and for P cells). There was no significant difference between the means ($P > 0.5$) at each voltage, but inactivation of the Na^+ current was significantly faster at -1.5 mV than at -11 mV ($P < 0.025$) in both R and P cells.

K^+ is reduced. Upon repolarization the K^+ tail currents should be inward, since E_K is now positive with respect to the holding potential of -50 mV. As expected, increasing external $[\text{K}^+]$ from 4 to 40 mmol l^{-1} reduced reversibly the peak outward current by nearly half (Fig. 5) and produced inward K^+ tail currents during repolarization to the holding potential of -50 mV.

Additional evidence for the role of K^+ was provided by reversibly blocking outward currents with $25\text{--}100 \text{ mmol l}^{-1}$ TEA^+ (solution 5), with $0.1\text{--}1 \text{ mmol l}^{-1}$ quinidine (added to solution 4) and with $5\text{--}10 \text{ mmol l}^{-1}$ 4-AP (solution 6, Table 1). Fig. 6 shows the effects of 30 mmol l^{-1} TEA^+ on outward current in an R cell (solution 5, Table 1). Similar blockade of outward currents by TEA^+ and

quinidine was seen in AP, T, P and N cells. 4-AP reduced K^+ currents in all five types of cells.

Distinctive characteristics of K^+ currents in AP, R and sensory neurones

As shown in Fig. 7, the outward K^+ currents in AP, R, P and T cells differed markedly. The currents were larger and faster in AP and R cells than in the sensory cells. To categorize the various K^+ currents the following measurements were made in each type of cell: (1) the rate of activation, which was not a simple exponential, was characterized by the $t_{1/2}$ to peak; (2) inactivation was described by its time constant and by the effect of various levels of steady holding potential; (3) Ca^{2+} -activated K^+ currents were identified by applying Ca^{2+} channel blockers.

Four types of K^+ current were distinguished; they have been designated as I_A (rapid activation and inactivation), I_C (Ca^{2+} -dependent), I_{K1} and I_{K2} (analogous to delayed rectifier K^+ currents of squid axon), in accordance with nomenclature

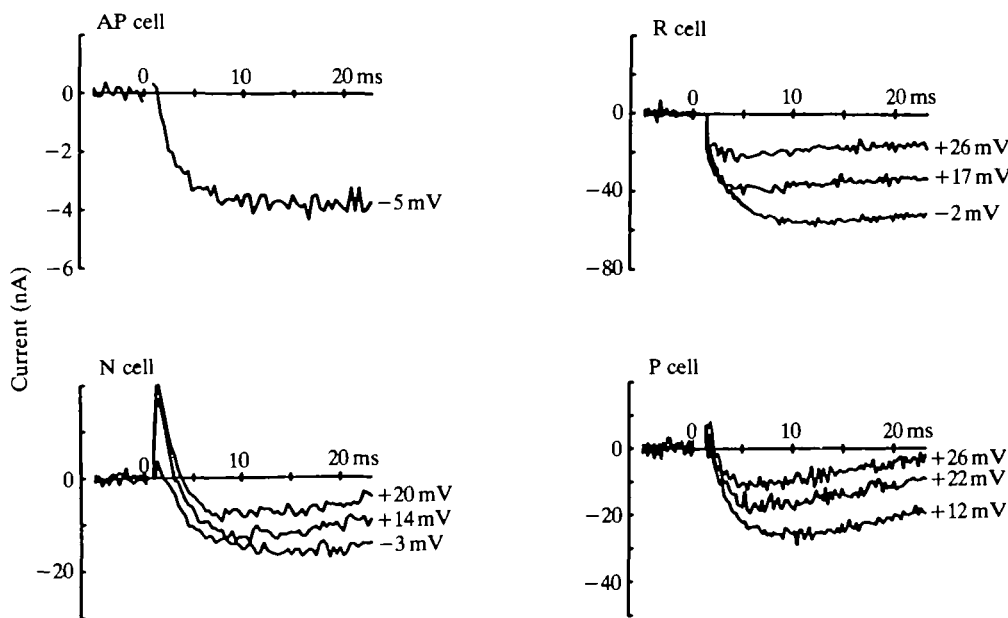


Fig. 4. Calcium currents in AP, R, N and P cells. AP cell: inward Ca^{2+} current evoked by voltage step from -40 to -5 mV. Recording solution contained $10 \text{ mmol l}^{-1} Ca^{2+}$ and $100 \text{ mmol l}^{-1} TEA^+$ and 5 mmol l^{-1} 4-AP to block K^+ currents (solution 13, Table 1). A holding potential of -40 mV was used to inactivate the remaining K^+ current of the A type. Current record is average of five traces. R cell: inward Ca^{2+} currents activated by voltage steps from -50 to -2 , $+17$ and $+26$ mV (solution 12, Table 1). Each current record is the average of four traces. N cell: Ca^{2+} currents activated by voltage steps from -50 to -3 , $+14$ and $+20$ mV (solution 9, Table 1). Each current record is the average of four traces. Outward Na^+ currents are present. P cell: Ca^{2+} currents activated by voltage steps from -50 to $+12$, $+22$ and $+26$ mV (solution 12, Table 1). Each current record is the average of four traces. Capacitative artefacts are not shown in these records.

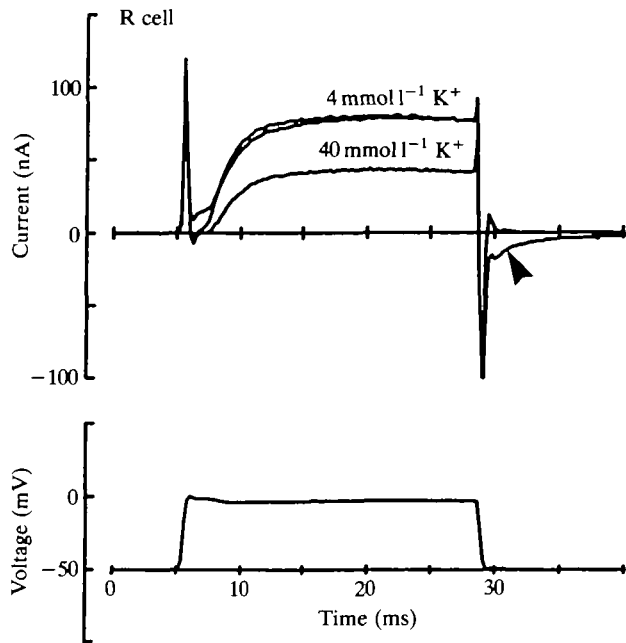


Fig. 5. Outward currents in an R cell were reversibly reduced by elevated external [K⁺] (40 mmol l⁻¹) and were unaffected by low external [Na⁺]. The two current records indicated by a 4 mmol l⁻¹ K⁺ are control traces before and after bathing the cell in 40 mmol l⁻¹ K⁺, low-Na⁺ medium (solutions 4 and 10, Table 1). The arrowhead designates inward tail currents recorded in 40 mmol l⁻¹ K⁺, low-Na⁺ medium. Inward Na⁺ currents were absent.

Table 2. Potassium currents in identified leech neurones

| Current | Activation | Inactivation | Steady-state inactivation* | Ca ²⁺ -dependence | Cell type |
|-----------------|--------------|--------------|----------------------------|------------------------------|-----------|
| I _A | Fast | Fast | Complete | None | AP,R |
| I _C | Fast | Intermediate | Little | Complete | R |
| I _{K1} | Intermediate | Slow | ND | None | P,N,T,R† |
| I _{K2} | Slow | Very slow | ND | ND | P,N,T,R |

* For steady-state inactivation, K⁺ currents evoked by voltage steps to 0 mV were compared at holding potentials of -70 and -30 mV.

† I_{K1} activated fastest in R cells followed in order by T, P and N cells.

ND, not determined.

established by others (Adams & Benson, 1985; Kaczmarek & Levitan, 1987). The currents are described for each type of cell in the following paragraphs and summarized in Table 2. Our principal emphasis has been to establish the distribution of the various currents in different cells rather than to define their kinetics and voltage-sensitivity in detail.

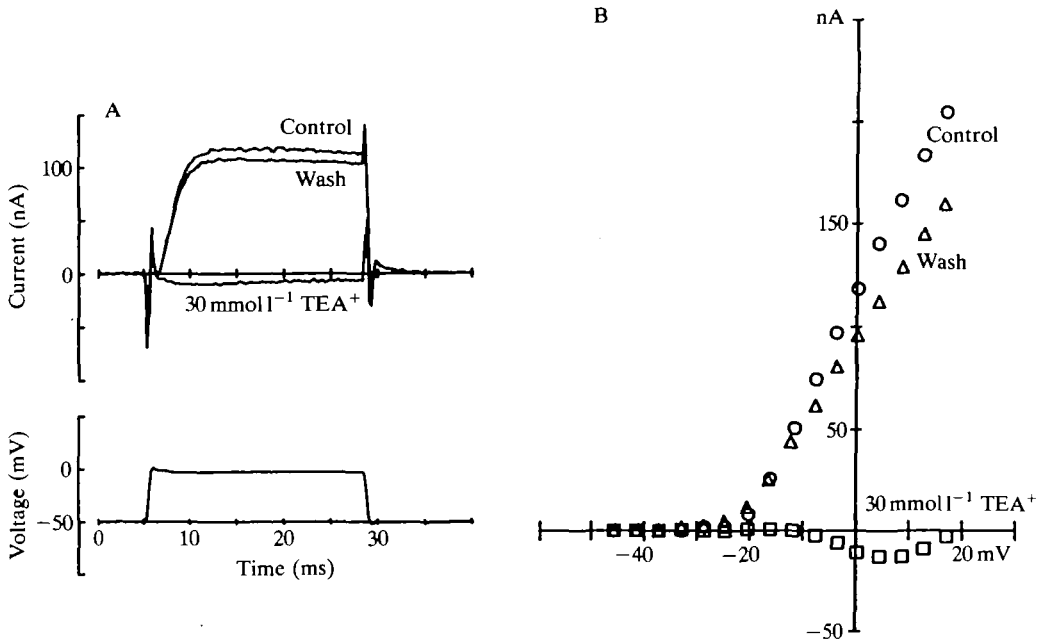


Fig. 6. Outward K^+ currents blocked reversibly by $30 \text{ mmol l}^{-1} \text{ TEA}^+$ to reveal an inward current. (A) Currents recorded during step depolarization to 0 mV . (B) Peak outward current (circles, triangles, no TEA^+) and inward current (squares, $+\text{TEA}^+$) plotted against voltage (solutions 4 and 5, Table 1).

R cell K^+ currents

Complex, intermingled K^+ currents with markedly different characteristics were apparent in R cells. It was of importance for studies in which R cells make synapses onto P cells and release 5-hydroxytryptamine (5-HT) to establish the identities of these currents and to determine how they could be selectively eliminated by pharmacological agents or by inactivation.

A major K^+ current was Ca^{2+} -activated and corresponded to I_C . Thus, the outward current was reduced by $64 \pm 5\%$ (S.E.M., $N=6$) in the presence of $100 \mu\text{mol l}^{-1} \text{ Cd}^{2+}$, or after substitution of Mn^{2+} for Ca^{2+} (solutions 7, 11 and 14, Table 1; Fig. 8A). Fig. 8B shows the N-shaped current-voltage relationship of K^+ currents in fluid containing $2 \text{ mmol l}^{-1} \text{ Ca}^{2+}$. After addition of $100 \mu\text{mol l}^{-1} \text{ Cd}^{2+}$, the outward current changed to a monotonic function with increasing steps of depolarization. The separation of K^+ currents in R cells, into Ca^{2+} -activated and Ca^{2+} -independent, was further supported by the measurements of activation shown in Fig. 8C. Again after addition of $100 \mu\text{mol l}^{-1} \text{ Cd}^{2+}$, the time to reach half-peak current was a simple monotonic function, activation becoming faster with larger depolarizing steps. In contrast, the relationship was complex under conditions allowing Ca^{2+} entry and activation of I_C . The time to half-peak decreased with steps of depolarization up to about $+30 \text{ mV}$ and then increased, presumably reflecting changes in the driving force on Ca^{2+} . I_C in R cells was not

blocked by high concentrations of charybdotoxin (95 nmol l^{-1}) or apamin ($10 \text{ } \mu\text{mol l}^{-1}$) which are peptide blockers of other Ca^{2+} -activated K^+ channels (Hugues *et al.* 1982; Miller *et al.* 1985).

At least three other K^+ currents with distinctive features could be recognized in R cells. As shown in Fig. 8, a K^+ current, designated I_{K1} , remained after blockage of I_{C} ; I_{K1} was slower than I_{C} and was blocked in the presence of $25\text{--}100 \text{ mmol l}^{-1}$ TEA⁺ (solutions 4 and 5, Table 1). A second, even slower current, I_{K2} , was observed during very long depolarizing pulses (1–2 s) in the presence or absence of I_{C} (Fig. 9). Increased external $[\text{K}^+]$ (from 4 to 23 mmol l^{-1}) reduced peak I_{K1} and I_{K2} currents as expected, and reversed the tail currents observed upon repolariz-

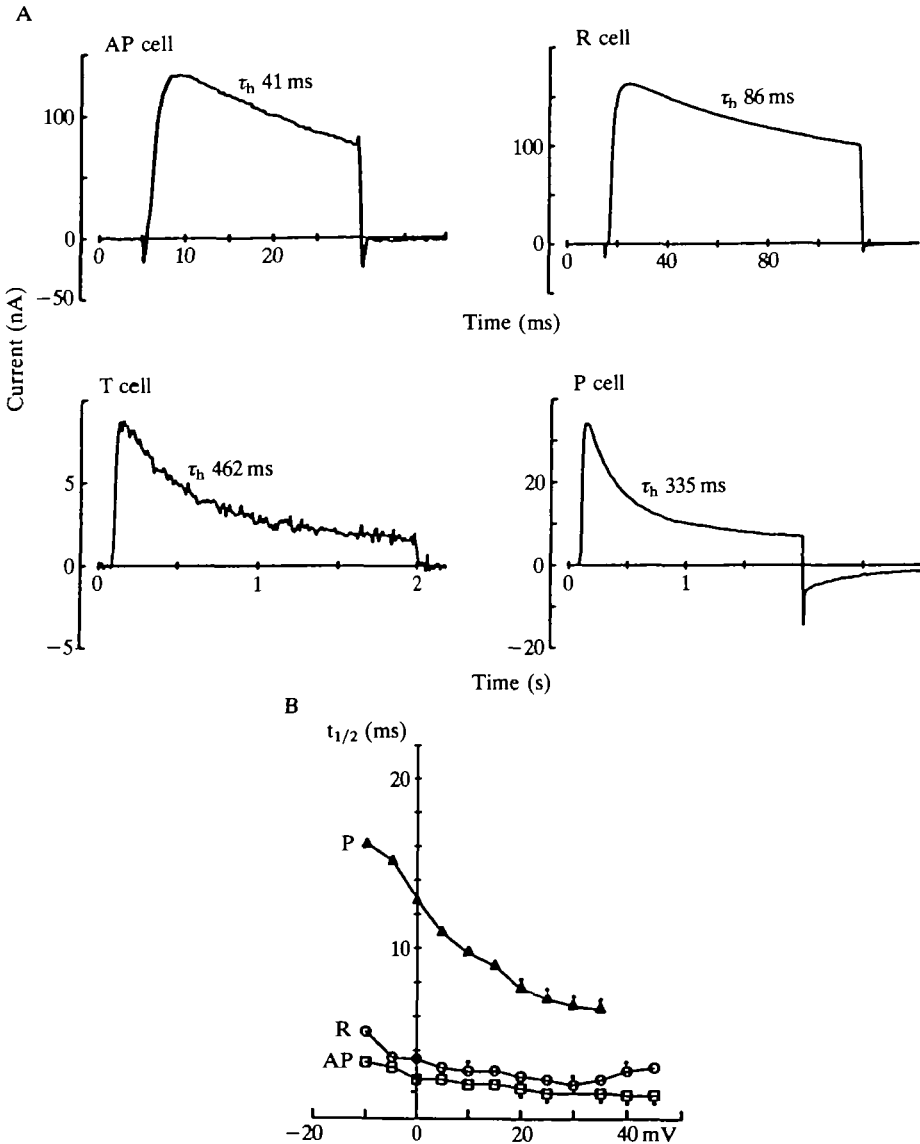


Fig. 7

ation to -50 mV. K^+ currents similar to I_{K1} and I_{K2} predominated in sensory T, P and N cells (see below).

In addition to I_C , I_{K1} and I_{K2} , a rapidly activating and inactivating A-type current (I_A) was revealed in R cells. This residual A-current was present after blockage of I_C , I_{K1} and I_{K2} (by low- Na^+ , Ca^{2+} -free, Mn^{2+} , TEA^+ fluid; solution 15, Table 1) and was reduced further by addition of 5 mmol l^{-1} 4-AP. It is described in greater detail for AP cells, below. Unlike the slower K^+ currents, I_A was completely inactivated at holding potentials more positive than -40 mV (see Fig. 10C).

K^+ currents in AP cells

The predominant K^+ current in AP cells was of the A-type (I_A). Blockage of Ca^{2+} entry did not decrease outward K^+ currents in AP cells as it did in R cells. Instead, a small increase in outward current was observed, presumably owing to the abolition of a competing inward Ca^{2+} current. Thus I_C appears to be absent from AP cells. Nor did we find any trace of outward currents that activated and inactivated slowly with kinetics similar to those of I_{K1} and I_{K2} .

Although activation of I_A in AP cells was rapid and similar to activation of outward K^+ current in R cells (Fig. 7B), inactivation of I_A was considerably faster. The time constant for inactivation (τ_h) for AP cells was 26 ± 2 ms (S.E.M., $N = 8$) compared with 97 ± 12 ms (S.E.M., $N = 10$) for R cells, when tests were made with prolonged steps from -60 to 0 mV.

I_A also showed pronounced steady-state inactivation. When the holding membrane potential was changed from -70 to -30 mV in an AP cell, the K^+ current evoked by a step to 0 mV was reduced by $81 \pm 6\%$ (S.E.M., $N = 6$). The absence of I_C , I_{K1} and I_{K2} currents in AP cells suggested that the small outward current that remained when holding at -30 mV represented I_A that had not been completely inactivated. It was, however, slightly slower in its activation than the major outward A-type current. Thus, for residual I_A $t_{1/2}$ of activation starting from -30 mV was 3.2 ± 0.5 ms (S.E.M., $N = 4$) compared with $t_{1/2}$ starting from -70 mV of 2.1 ± 0.18 ms (S.E.M., $N = 6$) for total I_A ($P < 0.05$). This slowing of activation

Fig. 7. (A) Characteristic K^+ currents recorded in AP, R, T and P cells. Inactivation time constants are indicated by τ_h . Holding potential was -50 or -60 mV and voltage was stepped to 0 mV. K^+ currents for AP, R and T cells were recorded in 4 mmol l^{-1} external K^+ (solution 4, Table 1). The K^+ current for the P cell was recorded in elevated external K^+ (23 mmol l^{-1}) and as a result the tail currents were inward (solution 8, Table 1). This result shows that K^+ accumulation did not produce the decline in outward current. Note the different time scales. (B) Voltage-dependence of time-to-half-peak amplitude ($t_{1/2}$) for K^+ currents in P (triangles), R (circles) and AP (squares) cells. Each symbol represents the average $t_{1/2}$ from five or more cells except for those indicated by the dots which represent average values from three or four cells. Clearly R and AP cells have K^+ currents that activate more rapidly than P cells. For P cells, standard deviations were at a maximum of 5.3 ms at -10 mV and at a minimum of 1.1 ms at $+15$ mV. The means (P versus R or AP) were significantly different at all voltage steps ($P < 0.005$, Student's *t*-test).

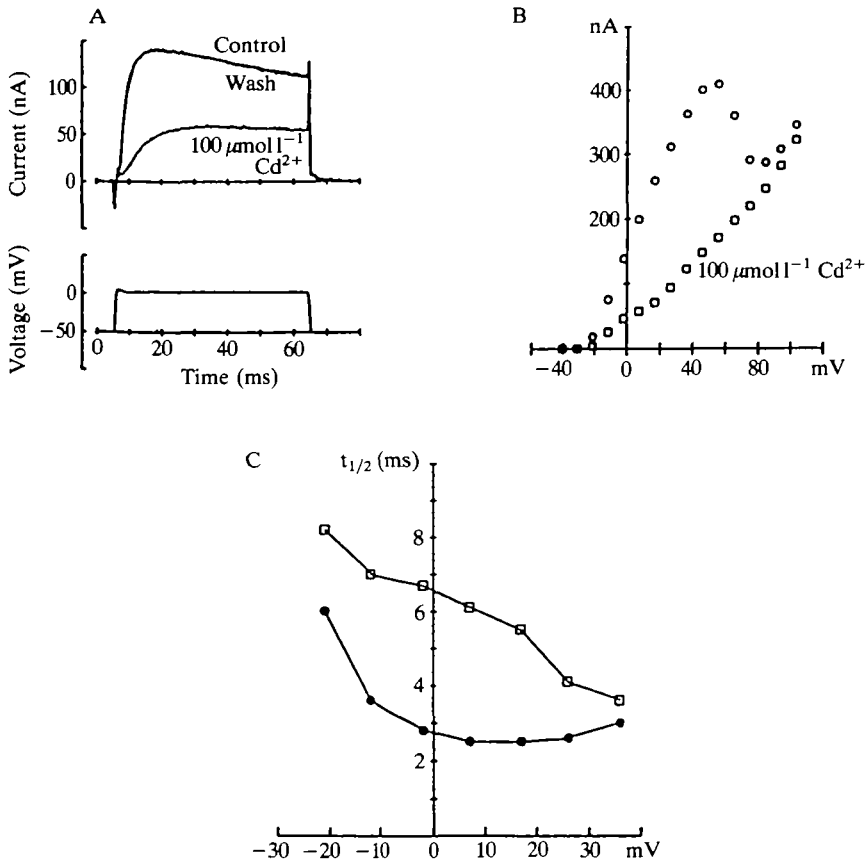


Fig. 8. Ca^{2+} -dependence of fast-activating K^+ currents (I_C) in Retzius (R) cells. (A) K^+ currents (top traces) recorded in low- Na^+ , normal- Ca^{2+} medium, with and without $100 \mu\text{mol l}^{-1} \text{Cd}^{2+}$ in the medium, are indicated by Control, $100 \mu\text{mol l}^{-1} \text{Cd}^{2+}$ and Wash (solutions 4 and 7, Table 1). Cd^{2+} decreased the amplitude and slowed the activation kinetics of the K^+ current. Outward currents were evoked by voltage steps from -50 to 0 mV. (B) Peak amplitudes of K^+ currents measured and averaged between 10 and 20 ms for control (circles) traces and between 20 and 30 ms for $100 \mu\text{mol l}^{-1} \text{Cd}^{2+}$ (squares) traces plotted against membrane voltage. The control current-voltage curve is N-shaped, a characteristic of Ca^{2+} -activated K^+ currents. Outward currents in $100 \mu\text{mol l}^{-1} \text{Cd}^{2+}$ were reduced in amplitude. (C) Voltage-dependence of time-to-half-peak amplitude ($t_{1/2}$) for K^+ currents in an R cell bathed in solutions containing $2 \text{ mmol l}^{-1} \text{Ca}^{2+}$ (filled circles) and $2 \text{ mmol l}^{-1} \text{Ca}^{2+}$ plus $100 \mu\text{mol l}^{-1} \text{Cd}^{2+}$ (open squares). Same cell as in A and B. Recorded in solutions 4 and 7 of Table 1.

appeared to be due to a competing inward Ca^{2+} current (see example of Ca^{2+} current in Fig. 4).

A comparison was made of the effects of membrane holding potentials on residual I_A of AP and R cells in solutions containing $100 \text{ mmol l}^{-1} \text{TEA}^+$ and $10 \text{ mmol l}^{-1} \text{Mn}^{2+}$ (Fig. 10C; solution 15, Table 1). The amplitude of I_A in both AP

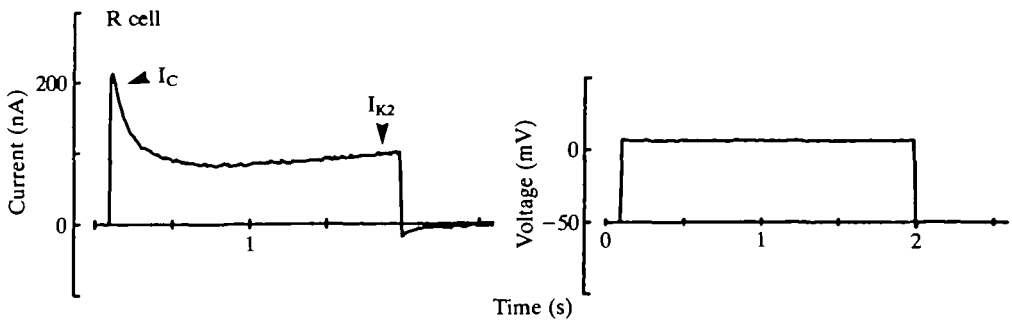


Fig. 9. I_C and very slowly activating K^+ current recorded in an R cell. The slowly activating K^+ current (I_{K2}) was present in many recordings from P, N and T cells, but not in AP cells. I_{K1} is not labelled since it cannot be distinguished in the presence of I_C and only becomes evident after I_C has been blocked. Recorded in solution 8 of Table 1.

and R cells behaved similarly and declined steeply at holding potentials between -50 and -35 mV.

K^+ currents in sensory cells

The K^+ currents in P, N and T cells were slower than those in AP and R cells (Fig. 7) and resembled the delayed rectifiers described for squid axons, designated as I_{K1} (intermediate) and I_{K2} (slower activation). I_{K1} predominated and in certain sensory neurones was the only detectable K^+ current. To measure activation and inactivation, depolarizing pulses were applied after the membrane potential had been held at various steady levels (from -70 to -40 mV). In P cells the half-time to reach peak ($t_{1/2}$) was 16 ± 2 ms (s.e.m., $N = 7$) for voltage steps to -10 mV. With larger steps of depolarization (to $+35$ mV) the half-time of activation decreased to 6 ms ($N = 3$; see Fig. 7B). Inactivation of I_{K1} in P cells was slow and followed an exponential time course ($\tau_h = 423 \pm 58$ ms; s.e.m., $N = 15$).

In N cells, activation and inactivation were even slower for voltage steps to 0 mV ($t_{1/2} = 31 \pm 5$ ms, s.e.m., $N = 5$; $\tau_h = 1700 \pm 280$ ms; s.e.m., $N = 7$). T cells had the fastest activation of sensory neurones for voltage steps to 0 mV ($t_{1/2} = 9$ ms, $N = 4$); inactivation, however, was similar to that of P cells ($\tau_h = 402$ ms, $N = 3$).

For reasons that are not clear, I_{K2} , the slowly activating K^+ current, was evident in only certain of the cultured neurones. I_{K2} was characterized by its very slow activation kinetics which caused the outward current to rise continually during a maintained pulse without reaching a plateau in 2 s. This was the longest pulse that could be applied without damaging the cell irreversibly. The slow progressive creep of I_{K2} activation was still present in raised external $[K^+]$ (23 mmol l^{-1}), showing that it was not caused by K^+ accumulation.

Discussion

In spite of the wealth of information about the electrical properties and

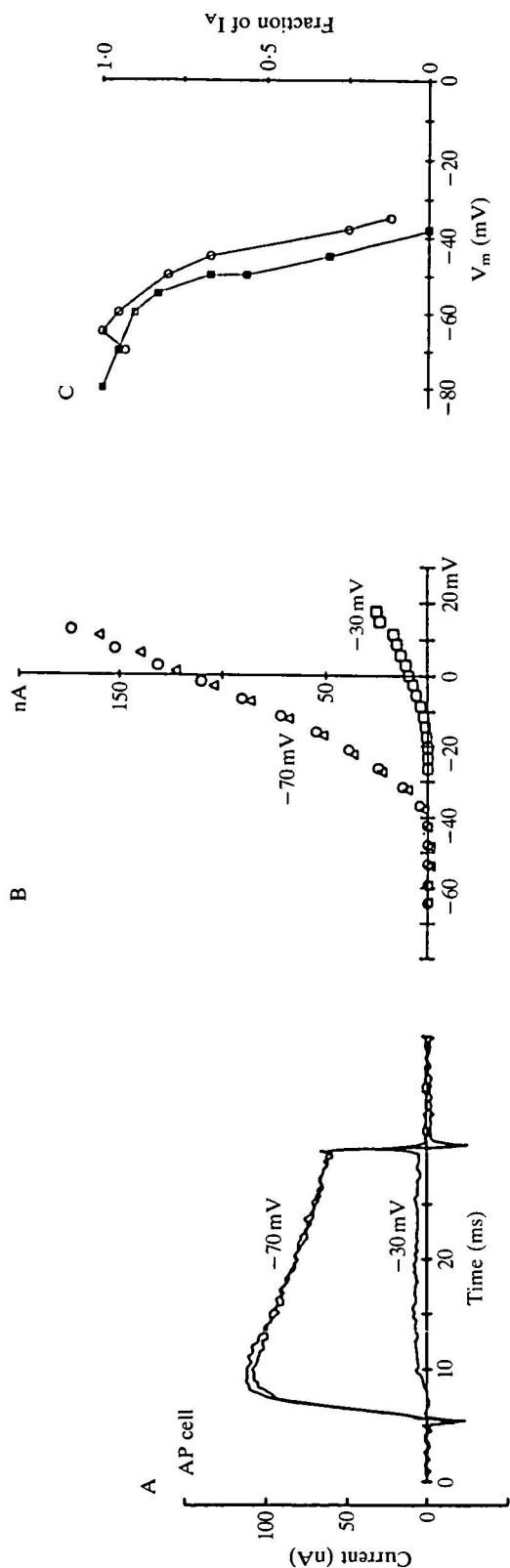


Fig. 10. Effects of holding potential on K⁺ currents in AP cells. (A) K⁺ currents evoked by voltage steps to 0 mV from holding potentials of -70 and -30 mV. Activation kinetics were slower for K⁺ currents evoked from -30 mV (solution 4 of Table 1). (B) Peak amplitudes of K⁺ currents plotted against command voltages. Control traces (circles, -70 mV) and partially inactivated traces (squares, -30 mV). K⁺ currents activated at -40 mV when held at -70 mV and at -15 mV when held at -30 mV. Same cell as in A. (C) Voltage-dependence of steady-state inactivation of I_A in AP and R cells. In both AP (open circles) and R (filled squares) cells, outward current remained after bathing the cells in 100 mmol l⁻¹ TEA⁺ (to block K⁺ currents), 10 mmol l⁻¹ Mn²⁺ (to block Ca²⁺ currents) and 10 mmol l⁻¹ Na⁺ (to reduce Na⁺ currents) (solution 15, Table 1). Voltage steps were to +10 mV from various holding potentials. V_m, membrane holding potential. Fraction of I_A, peak A current measured at each V_m and normalized relative to the largest A current measured. Different AP cell from the one shown in Fig. 10A and B.

signalling characteristics of identified neurones in the CNS of the leech *Hirudo medicinalis*, there has till now been no attempt to describe the major cationic currents in the various cell types. An exception is the analysis of K^+ currents with two-electrode voltage-clamp in Retzius cells in the CNS of a different leech, *Macrobdella decora* (Johansen & Kleinhaus, 1986). Technical difficulties have stood in the way of more comprehensive analyses of the distribution and the characteristics of Na^+ , Ca^{2+} and K^+ currents in leech neurones. First is the absence of a selective Na^+ channel blocker; instead of simply applying tetrodotoxin, the external Na^+ concentration must be lowered. Worse still are the relatively small sizes of the cells which make it necessary to impale them with two fine, high-resistance microelectrodes. As a result it becomes difficult to deliver sufficient current to control the membrane potential and the capacitative artefacts can encroach upon the early phases of activation, especially for Na^+ .

With such considerations in mind, the aims of the present experiments were limited. The use of an isolated neurone in culture offers the advantage that one can, with confidence, clamp the cell in its entirety and change its fluid environment rapidly. Conventional two-electrode voltage-clamp seemed suitable as a first step in comparing channel types and densities in those neurones most frequently studied in culture. The results presented here were not designed to measure precise kinetics of Na^+ , K^+ and Ca^{2+} activation and inactivation, for which other techniques such as patch-clamp, intracellular perfusion or loose-patch (Bookman & Dagan, 1987; R. J. Bookman & Y. Liu, in preparation; Bookman *et al.* 1987) can offer better temporal and spatial resolution. Rather, the aim was to establish whether different types of channels for cations existed in R, AP, P, N and T cells. These cells were selected because they show highly distinctive action potentials, delayed rectification and afterpotentials that are retained in culture. Moreover, in culture, extensive use has been made of R and AP cells to study neurite outgrowth and of R and P cells to study synapse formation (Nicholls, 1987).

Our results lead to certain clear conclusions. The time constants for inactivation of Na^+ currents (unlike the activation kinetics) could be measured accurately, as could maximum peak Na^+ current. It became evident that P and N cells, with their large action potentials, did not differ from R, T and AP cells with respect to Na^+ inactivation but only in the density of Na^+ channels. Similarly, what distinguished the Ca^{2+} currents from cell to cell was the peak amplitude rather than the rate of rise. For K^+ currents the picture was more complex and remains incomplete. Simplest were the AP cells with the fastest activating and inactivating K^+ currents. This, together with the minuscule Na^+ currents, can account for the small amplitude of impulses in the soma, as well as the high rates of firing and the absence of delayed rectification that together identify the AP cell electrically.

The analysis is less satisfactory for the sensory cells. For convenience and by analogy we have labelled the K^+ currents as I_{K1} and I_{K2} . These currents were far slower and could help to explain the larger sizes of action potentials, the undershoot, and the delayed rectification in T, P and N cells. Activation of I_{K1} was fastest in T cells, followed by P and then N cells. These differences in activation

probably correlate with the rapid repolarization of action potentials of T cells compared with the slow repolarization of N cells. But we cannot account for these phenomena quantitatively and numerous other anomalies remain. For example, no evidence for Ca^{2+} -activated K^+ current was detected in P or N cells; yet these cells show a persistent hyperpolarization that depends on Ca^{2+} and long-lasting changes in G_K following trains of impulses (Jansen & Nicholls, 1973). A peculiarity that may be related concerns the very slow activation of I_{K2} which was seen in some P and N cells, but not others, in a manner not correlated with other variables.

Currents resembling I_{K1} and I_{K2} of leech sensory cells have been found in various different proportions in neurones of other invertebrates (e.g. see fig. 13 of Aldrich *et al.* 1979). One mechanism for such variability is thought to be phosphorylation of delayed rectifier K^+ channels through second-messenger systems. For example, increasing cyclic AMP levels in bag cells of *Aplysia* partially eliminates the fast-activating delayed rectifier K^+ current and completely eliminates the slowly activating K^+ current (Strong & Kaczmarek, 1986). It remains to be determined whether comparable K^+ channel regulation also occurs in leech neurones.

Two pairs of homologous N cells exist in each midbody ganglion of *Hirudo medicinalis*: a lateral and a medial pair. Lateral and medial N cells are similar in their functions and action potential shapes, but are different in that each type of cell exhibits specific antigens (Johansen *et al.* 1984a; Zipser, 1982; Blackshaw, 1982) and extrasynaptic receptors (Sargent, 1977; Johansen *et al.* 1984b). In our experiments we used N, T and P cells without taking account of subtypes. It could therefore be that analysis of K^+ currents in lateral and medial types of N or P cells might show subtle differences.

R cell K^+ currents were more complex and their functional significance harder to interpret. Not only were all the K^+ currents of the other types of cells displayed but there was an additional major Ca^{2+} -activated component. To what extent I_C , I_A , I_{K1} and I_{K2} currents represent different populations of channels with distinctive characteristics could not be resolved in our experiments in which separation of the component K^+ currents was achieved by drastic changes in ionic, TEA^+ and 4-AP concentrations.

At the same time these measurements set the stage for more detailed studies of synaptic transmission by R cells in culture. Synapses made by R cells upon P cells develop rapidly and show properties such as facilitation, depression and dramatic effects of steady holding potential on the release of 5-HT (the Retzius cell transmitter). It now becomes practicable to measure, under conditions defined by the present experiments, Ca^{2+} currents that are relatively uncontaminated by I_A , I_C or Na^+ currents, and to correlate Ca^{2+} entry with transmitter release during facilitation.

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