Properties of Internally Perfused, Voltage-Clamped, Isolated Nerve Cell Bodies

KAI S. LEE, NORIO AKAIKE, and ARTHUR M. BROWN

From the Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550

ABSTRACT The membrane properties of isolated neurons from *Helix aspersa* were examined by using a new suction pipette method. The method combines internal perfusion with voltage clamp of nerve cell bodies separated from their axons. Pretreatment with enzymes such as trypsin that alter membrane function is not required. A platinized platinum wire which ruptures the soma membrane allows low resistance access directly to the cell's interior improving the time resolution under voltage clamp by two orders of magnitude. The shunt resistance of the suction pipette was 10–50 times the neuronal membrane resistance, and the series resistance of the system, which was largely due to the tip diameter, was about 10^5 Ω. However, the peak clamp currents were only about 20 nA for a 60-mV voltage step so that measurements of membrane voltage were accurate to within at least 3%. Spatial control of voltage was achieved only after somal separation, and nerve cell bodies isolated in this way do not generate all-or-none action potentials. Measurements of membrane potential, membrane resistance, and membrane time constant are equivalent to those obtained using intracellular micropipettes, the customary method. With the axon attached, comparable all-or-none action potentials were also measured by either method. Complete exchange of Cs^+ for K^+ was accomplished by internal perfusion and allowed K^+ currents to be blocked. Na^+ currents could then be blocked by TTX or suppressed by Tris-substituted snail Ringer solution. Ca^{2+} currents could be blocked using Ni^{2+} and other divalent cations as well as organic Ca^{2+} blockers. The most favorable intracellular anion was aspartate$^-$, and the sequence of favorability was inverted from that found in squid axon.

INTRODUCTION

The voltage-dependent conductances of nerve cell bodies appear to be more numerous than those of their attached axons. In addition to the Na^+ and K^+ currents found in axons (Hodgkin and Huxley, 1952), the following voltage-dependent currents have been reported in molluscan neurons: a transient Ca^{2+} current (Geduldig and Gruener, 1970; Kostyuk et al., 1974a, 1974b; Standen, 1975b); a persistent, slowly inactivating Ca^{2+} current (Eckert and Lux, 1976); a fast transient K^+ current (Hagiwara et al., 1961; Connors and Stevens, 1971; Neher, 1971); an anomalously rectifying K^+ current (Kandel and Tauc, 1964); a slow K^+ current (Brodwick and Junge, 1972; Partridge and Stevens, 1976); and

a Ca^{2+}-activated K^+ current (Meech and Standen, 1975; Heyer and Lux, 1976; Brown et al., 1977). These conductances have not been characterized as completely as those in axons (Hodgkin and Huxley, 1952) because of two difficulties: separation and spatiotemporal resolution of the ionic currents. This is unfortunate because, judging from the example of squid giant axon, knowledge of the voltage and time-dependence of individual conductances would provide insight into basic ion conduction processes as well as the framework for an explanation of the wide repertoire of neuronal functions such as spontaneous discharge, extended ranges of discharge frequency, subthreshold oscillations, and long-lasting changes in membrane potential. We have developed a method that overcomes the difficulties mentioned above. The method combines voltage clamp and internal perfusion of isolated nerve cell bodies without exposing neurons to enzymes that are known to alter membrane function. In the present paper, the method is evaluated and the properties of neurons examined with it are described. In the accompanying paper (Akaike et al.) the method was used to separate and analyze the Ca^{2+} current of neurons.

METHODS

Preparation

The experiments were done on neurons located in the subesophageal ganglion of *Helix aspersa*. The ganglion was removed and the connective tissue was stripped off with jeweller's forceps until clusters of neurons floated free in the snail Ringer (composition shown in Table I). Individual neurons of $\cong 100$ μm in diameter were then partially aspirated under a negative pressure of about -30 mm Hg so as to occlude the 15–50-μm diameter tip of a suction pipette (Fig. 1). The suction pipette was pulled from Pyrex tubing 3.0 mm OD and tapered over a length of 0.5–1.0 cm from its tip. The wall thickness at the tip was about 10 μm and the volume of the pipette was about 5 ml. The pipette was coupled to a sliding micromanipulator (Carl Zeiss, Inc., New York) which was moved so as to rend the neuron free from both its neighbors and its axon. The experiments were done at room temperature of 18–20°C.

Intracellular Perfusion

Internal perfusion was preceded by disrupting part of the neuronal membrane aspirated into the tip of the suction pipette. This was accomplished by inserting manually a platinized platinum wire having a diameter of 1–2 μm at its tip through the membrane into the cytoplasm. The tip was sharpened electrolytically using pure platinum wire 20 μm in diameter and a solution of 95 ml H_3PO_4, 5 ml H_2SO_4, and 1 g gelatin. An alternative arrangement was to have the platinum wire at the tip of the pipette so that aspiration was accompanied by disruption. The wire which had a resistance of 30 kΩ was also used to pass current directly into the cell (Fig. 1). The inlet tubing for perfusion (Fig. 1) was positioned near the membrane whereas the outlet tube connected to the suction was more remote. Inflow was delivered from a group of parallel reservoirs which were usually $\cong 30$ cm above the neuron. Negative pressure was applied via a series of trap bottles and was adjusted so as to obtain a minimum of damage and a maximum seal from the extracellular fluid. The negative pressure together with the hydrostatic pressure resulted in a flow rate through the suction pipette of 0.5 ml/min. The composition of the internal perfusion fluids is given in Table I.

TABLE I
SNAIL EXTERNAL AND INTERNAL SOLUTIONS

	NaCl	Tris* Cl	KCl	CsCl	CaCl₂	MgCl₂	K aspartate	Cs aspartate
					mM			
External solution (pH 7.6)								
Normal	75	5	5	—	10	15	—	—
Tris	—	80	5	—	10	15	—	—
Tris, Cs	—	80	—	5	10	15	—	—
Internal solution (pH 7.4)								
K aspartate	—	—	—	—	—	—	105	—
Cs aspartate	—	—	—	—	—	—	—	110
KCl	—	—	105	—	—	—	—	—

pH in each solution was adjusted by Tris* base.
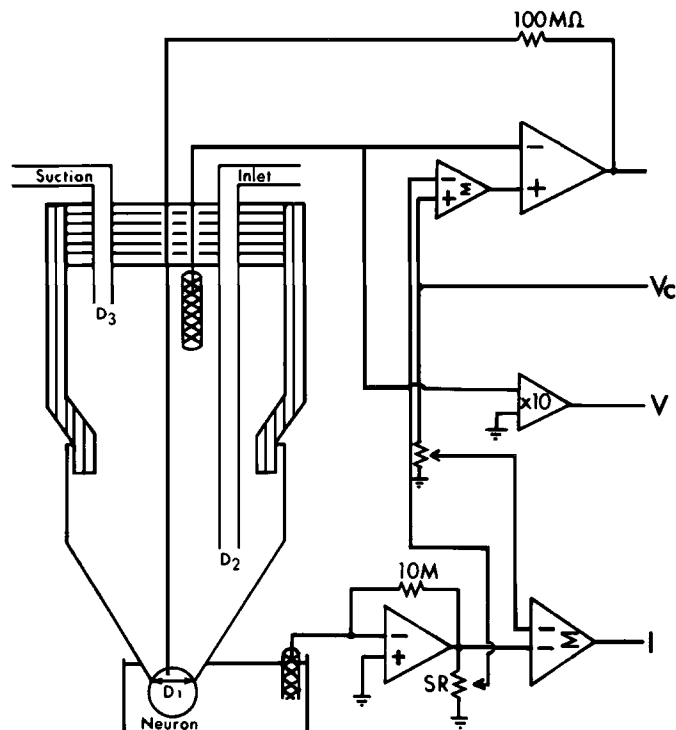* Tris: tris (hydroxymethyl) aminomethane.

FIGURE 1.   Experimental arrangement. Drawing not to scale. D_1, D_2, and D_3 are 25 µm, 500 µm, and 2 mm, respectively. The agar plug is cross-hatched. SR, series resistance compensation; M, megaohm.

Electrical Measurements

Membrane potential was measured using an Ag-AgCl wire about 1 mm in diameter at its tip inserted into a polyethylene tube 1 cm in diameter, filled with a 0.15 M KCl-agar plug which was placed inside the suction pipette (Fig. 1). The reference electrode was also an

Ag-AgCl wire connected to the bath by an 0.15 M KCl-agar bridge. The resistance of either electrode in snail Ringer was 1–2 kΩ and the resistance between the suction pipette with a 50-μm tip and the bath electrode was 100 kΩ. Both electrodes were connected via matched unity gain operational amplifiers (not shown) to a ×10 differential amplifier. Liquid junction potentials for the various internal and external solutions to which either electrode was exposed (Table I) were measured using an Ag-AgCl wire in 0.15 or 3.0 M KCl solutions as a reference. These potentials had values <2.0 mV.

Constant current pulses were passed from a stimulator (Digitimer, W-P Instruments, Inc., New Haven, Conn.), across a 5 × 10^{-8} Ω resistor to the bath.

The arrangement used for voltage clamping is shown in Fig. 1. The loop gain of the clamp amplifier at 50 kHz was 300, and the rise time to 90% for the voltage step was 10 μs. Series resistance compensation was used in some experiments according to the methods of Hodgkin et al. (1952). Series resistance was estimated from the clamp current transient and from the instantaneous voltage displacement produced by a step of current (Hodgkin et al., 1952). A circuit (not shown) was used to prevent damage from oscillations in clamp current. Current was measured using a current-to-voltage converter. It was also convenient in some experiments to nullify the current across the shunt resistance which isolated the aspirated part of the cell from the surrounding extracellular fluid.

Evaluation of the Method

Measurements of resting membrane potential, membrane resistance, action potential, current clamp voltage responses, and voltage clamp current responses were compared with measurements obtained using intracellular micropipettes, the standard technique. The micropipettes were filled with 3 M KCl, had tip diameters of 1 μm, resistances of 2–5 × 10^6 Ω, and were inserted into a partially aspirated cell.

The most important electrical parameters to be evaluated were the shunt and series resistances. The shunt resistance isolates the neuron from the bathing fluid and was estimated as follows. During isolation, voltage pulses were passed between the bath ground and the voltage electrode in the suction pipette. Isolation was usually successful when the current responses were reduced between 20- and 80-fold, and further increases in negative pressure had no additional effects (Fig. 2 A). Shunting around inserted micropipettes could be evaluated in the same way. Another simple test of satisfactory isolation was the amplitude of the action potential which should be similar to values obtained with intracellular micropipettes. A more accurate determination was made by inserting two micropipettes to inject transmembrane current and measure transmembrane voltage. The voltage deflections of the micropipette and of the voltage-sensing electrode in the suction pipette were compared before and after puncture of the aspirated portion of neuronal membrane (Fig. 5).

The adequacy of the internal perfusion system was assessed by measuring the intracellular K$^+$ and Cl$^-$ activities after changes in the internal perfusate. Intracellular K$^+$ and Cl$^-$ liquid ion exchanger microelectrodes were fabricated and used according to Russell and Brown (1972 a, b).

The leakage resistance was determined by measuring the voltage deflections produced by small hyperpolarizing pulses from the resting potential and from the current-voltage relationship that remained after blockage of all of the voltage dependent ionic conductances.

RESULTS

Separation of the Neuron

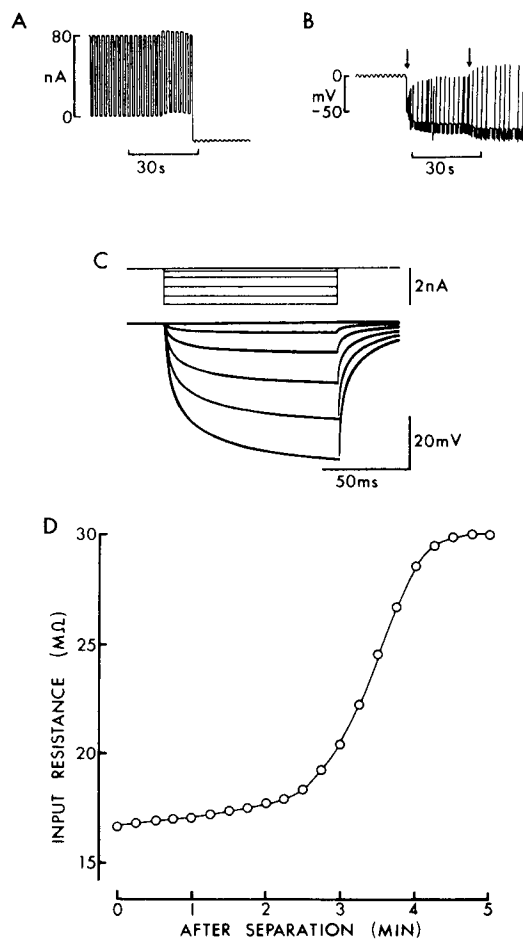As the suction pipette makes contact with the neuron, the current deflections

FIGURE 2. Separation of neurons. (A) Effects of aspiration on the currents elicited by constant voltage pulses passed between the suction pipette's interior and the bath ground. The marked reduction in current indicates good isolation. (B) Voltages elicited by constant current stimuli during isolation of a neuron. At the first arrow, a burst of abortive action potentials appear as the neuron is aspirated by the suction pipette. At the second arrow the aspirated membrane is ruptured by the current-passing wire. The voltage becomes progressively more negative reflecting the neuron's membrane potential E_m, and the hyperpolarizing voltages elicited by the constant current pulses become progressively larger. E_m is -60 mV. Full-blown action potentials with overshoots of $+20$ to $+30$ mV occur at the termination of each voltage pulse. (C) Voltage responses to constant current pulses in a neuron isolated from its axon. (D) Time-course of recovery of input resistance after membrane puncture.

elicited by constant voltage pulses are markedly reduced, and a steady current flows from pipette to bath (Fig. 2 A). When a micropipette is inserted into the cell, the current deflections under similar conditions are not reduced as greatly. The changes in suction pipette voltage with respect to the bath are shown in Fig.

2 B. Initial aspiration produces a negative potential, a transient burst of action potentials, and an increase in the voltage deflections elicited by constant current pulses. After disrupting the aspirated membrane with the Pt-Pt current wire, an increase in resting potential to about −60 mV is recorded (Fig. 2 B), and full-blown overshooting action potentials are elicited at the anodal breaks. At this stage suprathreshold depolarizing current pulses elicit all-or-none action potentials with overshoots of +30 to +40 mV (Fig. 3 A). The spikes arise from the axon and in voltage clamp axonal currents are seen at suprathreshold voltages (Fig. 3 C). The values for resting potential, action potential amplitude, and as we shall see later, membrane resistance are equivalent to the values obtained with intracellular micropipettes. To rend the soma free of its axon the micromanipulator, with its attached suction pipette, is moved. The resting potential is transiently reduced and then recovers within 5–10 min. Hyperpolarizing voltage responses with the time constant characteristic of transmembrane voltage changes are elicited by hyperpolarizing current steps (Fig. 2 C). The input resistance is always increased after recovery from rupture reaching values of 20–50 MΩ (Fig. 2 D), and damaged but persistent axonal remnants are not likely to be present. Most of the cells are no longer capable of producing all or none action potentials (Fig. 3 B). The axonal currents which invaded the soma formerly are no longer present, and smaller soma currents are observed (Fig. 3 D). The progression of inward soma currents with depolarizing steps is shown more completely in Fig. 9 A. Note that the ratio of inward-to-outward axonal currents is much greater than the ratio of inward-to-outward somal currents.

Adequacy of Internal Perfusion

This was evaluated directly by following the changes in concentration of K_i^+ or Cl_i^- when the internal perfusion fluid was switched to K^+-free or Cl^--rich solutions (Fig. 4). In the case of Cl^-, the neuron was perfused with K^+ aspartate (Table I), which was then changed to KCl. Within 2–3 min the intracellular Cl^- had risen from a few mM to 105 mM. When the aspartate perfusate was reinstituted, a quick fall in Cl_i^- occurred. The few mM Cl_i^- present in K aspartate reflects the selectivity of the Cl^- electrode (Cl^-: aspartate$^-$ selectivity ratio is 20–50).

The measured changes of K_i^+ were similar. Replacing K aspartate with Tris Cl resulted in a fall of K_i^+ from 105 mM to 10 mM within periods ranging from 2.5 to 5 min. The 10 mM residual levels of K^+ in Tris perfusate reflect the selectivity of the K^+ electrode (about 10–30 to 1 for K^+ over Tris$^+$).

The exchange times are affected by the shape of the suction pipette. The most rapid exchanges occur when the tip of the pipette has a very shallow taper.

Another method of evaluating internal exchange was to follow the blockage of the outward clamp currents after switching the internal perfusion fluid from K aspartate to Cs aspartate. The outward currents were usually completely blocked within 5–10 minutes of such a solution change (Fig. 10).

Evaluation of the Shunt and Series Resistances

The shunt resistance (R_{sh}) was calculated from data obtained by using the experimental procedure shown in Fig. 5 A. Membrane resistance was measured

by using intracellular micropipettes for passing transmembrane current and recording transmembrane voltage. The voltage deflections in the suction pipette were measured before and after perforation of the aspirated somal membrane. The equivalent electrical circuits are shown in Fig. 5 B (before puncture) and 5 C
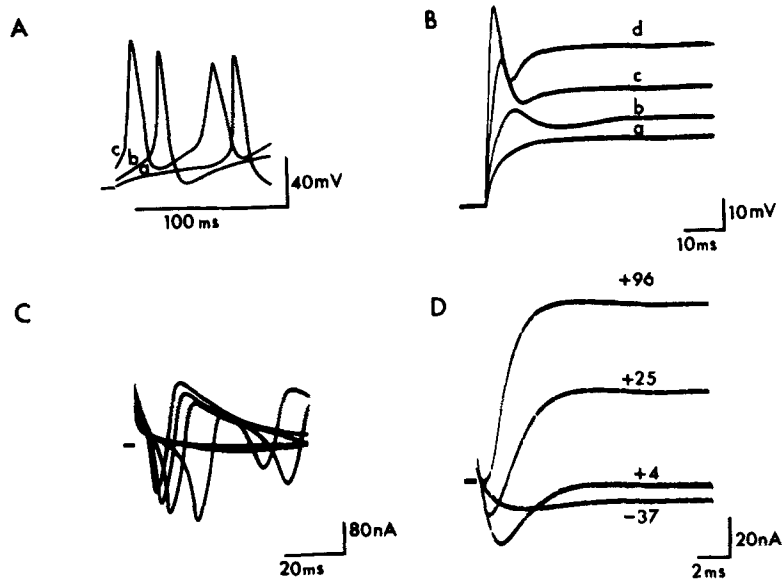

FIGURE 3. (A, B) Action potentials elicited by currents of 1, 2, 3, and 4 × 10^{-9} A in a, b, c, and d, respectively. (A) The low threshold spikes (E_m = -50 mV) originate from the axon and (B) are not seen after rending the neuron free from its axon (E_m = -60 mV). (B) The neuron has been rended free from its axon and healed over. All-or-none action potentials are no longer elicited, and the graded voltage-dependent responses require higher currents than when the axon is attached. (C) Voltage clamp currents from a neuron with attached axon. Currents are corrected for leakage in these and subsequent records except where stated otherwise. The voltage steps were to -45, -40, -39, -33, -23, and -4 mV and were applied from holding potential, V_h of -60 mV. The inward currents arise after a considerable latency and increase very little with increasing voltage clamp steps. They arise from the axon and dominate any somal currents. Note the repetitive responses at larger depolarizations indicating inadequate spatial control of voltage. (D) Voltage-clamp currents after tearing the neuron soma off its axon. V_h = -60 mV. The voltages of the steps are given for each current.

(after puncture). Before puncture the relevant equations are:

$$I = V_1/R_m + V_2/R_{sh} \qquad (1)$$

and

$$I_p = (V_1 - V_2)/R_p = V_2/R_{sh} = I_{sh}, \qquad (2)$$

where I is the current injected from the micropipette, V_1 is the transmembrane voltage change, V_2 is the voltage change in the suction pipette, I_p and I_{sh} are the
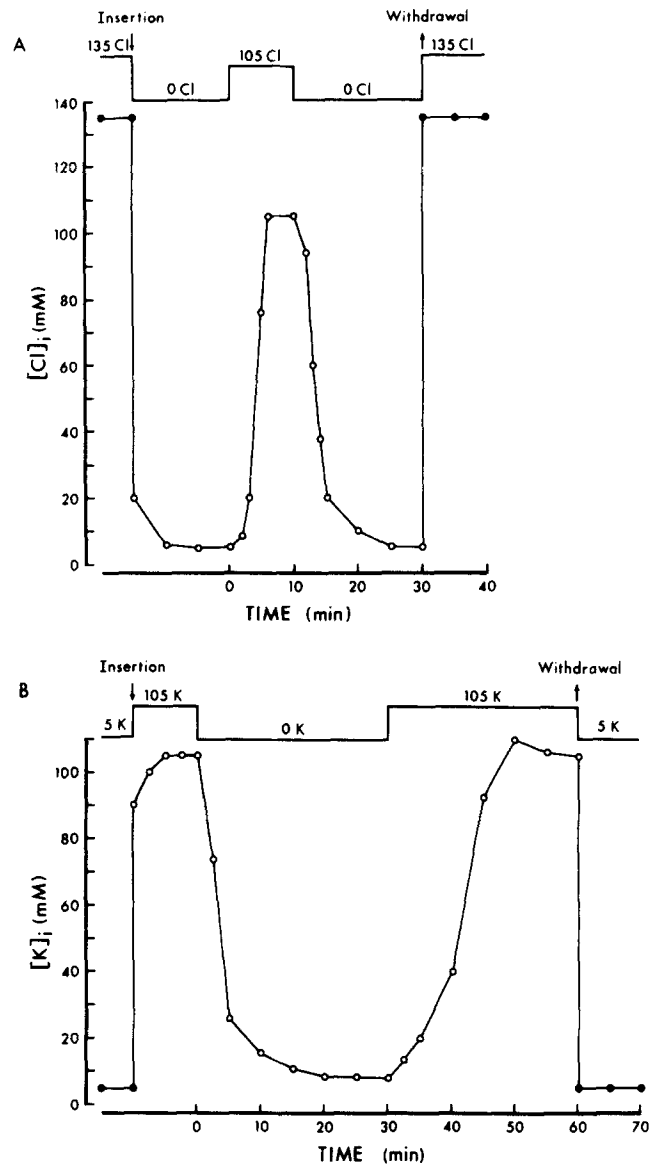
FIGURE 4.   (A) Changes in intracellular Cl^- concentration after changes in internal perfusate Cl^-. The concentrations in A and B were calculated from the measured ionic activities by using a measured activity coefficient of 0.77. At the first arrow the Cl^- microelectrode is inserted into the neuron which was perfused with K aspartate. 105 mM Cl^- was then substituted temporarily for aspartate⁻. (●) Extracellular and (○) intracellular Cl activities. (B) Changes in intracellular K^+ concentration after changes in internal perfusate K^+. Cs^+ was substituted for K^+. Sequences as in A.

patch and shunt currents, and R_m, R_{sh}, and R_p are the membrane, shunt, and patch resistances, respectively. After puncture we have:

$$\frac{V_3}{I} = \frac{R_m \cdot R_{sh}}{R_m + R_{sh}},$$

(3)

where V_3 is the voltage change in the suction pipette elicited by the same transmembrane current. In using this method to evaluate the system's electrical properties we have found that R_m ranges from 20 to 50 MΩ and R_{sh} has values of 10–50 × R_m.

The same arrangement allowed us to compare the current clamp responses of the suction pipette and micropipette systems using an artificial cell made of passive components which simulated the equivalent circuit of Fig. 5, but also included a parallel capacitor of 5 × 10^{-10} F. The cell was grounded, and current and voltage connections between it and either of the two pipette systems were made through the bath. The voltage responses to hyperpolarizing current steps
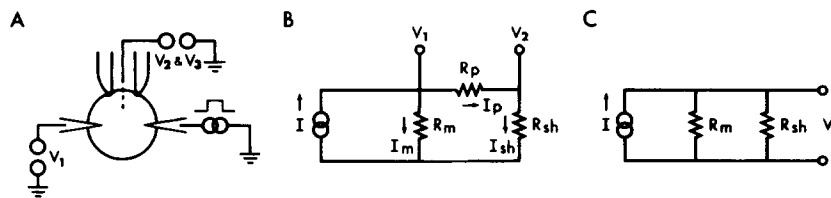

FIGURE 5. Evaluation of shunt resistance, R_{sh}, membrane resistance, R_m, and patch resistance, R_p. (A) Experimental procedures. (B) Equivalent circuit before puncturing the aspirated membrane. (C) Equivalent circuit after puncture.

were similar in amplitude and time-course. The current responses to voltage clamp steps were not. The rise times of the voltage steps for an unshielded micropipette system and for the suction pipette were 2 ms and 10 μs, respectively. The time constants for the respective current transients were 3–7 ms and 100–500 μs, respectively. As we shall see subsequently, the values for the suction pipette depend largely upon its tip diameter.

The current and voltage clamp responses of the same neuron examined with both methods were also compared (Fig. 6). The voltage responses to hyperpolarizing current steps were similar in amplitude and time-course as were the action potentials elicited by depolarizing current pulses (Fig. 6 B and C), but the similarities depended upon the tip diameter of the suction pipette and the resultant resistance to current flow at this site. The relationship is shown in Fig. 7 A; this resistance can be the main source of series resistance using the suction pipette method. If there is a sufficient IR or voltage drop across the tip during a step of current, a corresponding voltage deflection at $t = 0$ results, and this may cause the voltages recorded by the suction pipette to be greater than those recorded by intracellular micropipettes. This problem can be avoided by using appropriate tip diameters, but it can also be readily recognized and corrected for. In the case of voltage clamp, series resistance compensation (Fig. 1) may be used when unusually large currents (50–100 nA) occur.

In our experiments the voltage deflections elicited by steps of current at $t = 0$ usually gave values for the series resistance of 10^5 Ω. The clamp current transient was fitted by a single exponential (Fig. 7) and had time constants ranging from 150 to 500 μs. The series resistance measured from the transient according to Hodgkin et al. (1952) also had values of 10^5 Ω (Fig. 7). Series
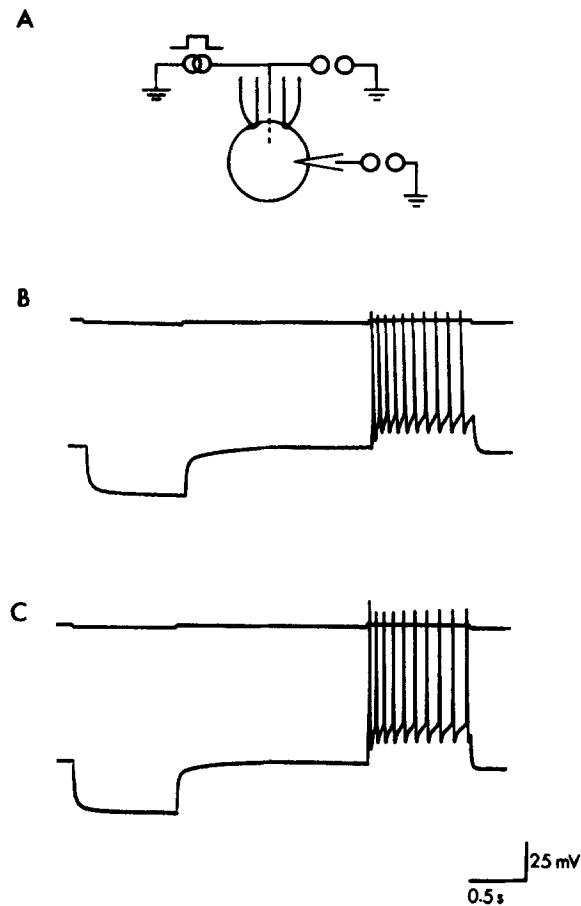

FIGURE 6. Comparison of successive voltage responses in neuron using the arrangement shown in A recorded by (B) an intracellular micropipette and (C) the suction pipette. Constant current pulses of 2 nA were used.

resistance compensation under these circumstances had little effect on the inward currents which were usually $<20 \times 10^{-9}$ A for voltage steps of about 70 mV. The membrane capacitance was calculated from the integrated clamp current transient and ranged from 300 to 600 pF. The lower values correlate reasonably with calculated values assuming a spherical cell body without significant infoldings of $\cong 100$ μm in diameter corrected for $\cong 20\%$ membrane loss with the suction pipette and a specific capacitance of 10^{-6} F cm^{-2}. The higher values may have been associated with larger cells but this was not examined.

Evaluation of the Leakage Resistance

The leakage resistance, R_l, was determined over a limited range in five experiments using intracellular micropipettes and the suction pipette on the
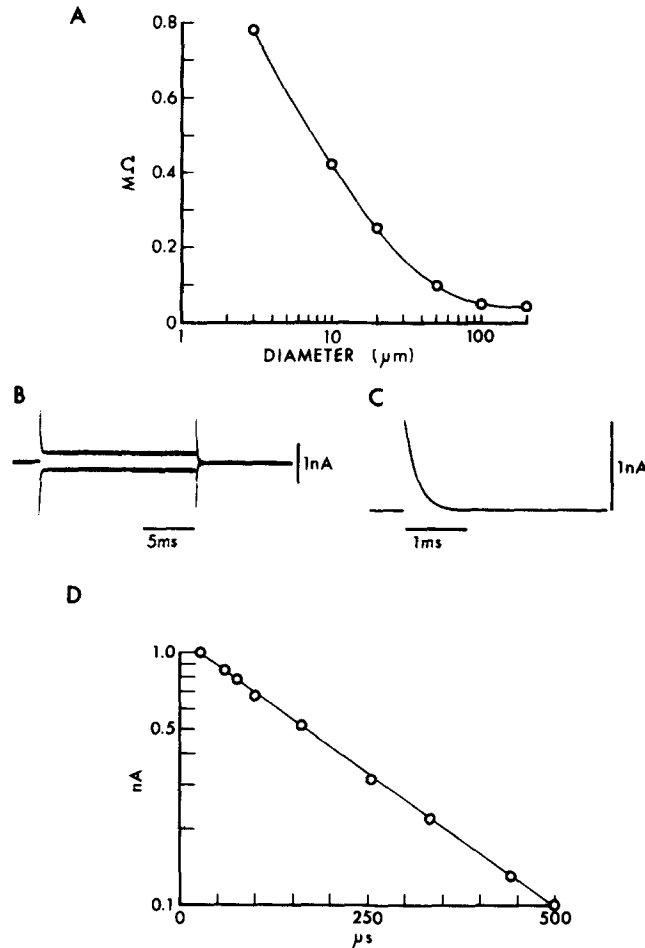

FIGURE 7. Evaluation of series resistance, R_s. (A) Relationship of suction pipette tip diameter to resistance of suction pipette. The IR drop across the tip was recorded as the voltage within the pipette when current was passed between the pipette and the bath. Most of our pipettes had tip diameters between 20 and 50 μm. (B) Current transients produced by small hyper- and depolarizing voltage steps. (C) Faster time base recording of the current transient elicited by the depolarizing step in B. The capacitative current is a single exponential function (D) and from its time constant a value of 10^5 Ω for R_s may be calculated.

same neuron in normal Ringer solution. It was calculated from the I-V relationship of small voltage pulses which were hyperpolarizing relative to the holding potential of -60 mV and gave equivalent values using either method although correction for the small contribution of R_{Sh} would give slightly higher

R_l's for the suction pipette method (Fig. 8). However, it is important that the leakage currents be evaluated at large depolarizing potentials because, as the following paper demonstrates, voltage-dependent inward currents become very small at such voltages. To do this, the K^+ currents were blocked with Cs^+ replacing K^+ in the internal and external solutions, the Na^+ currents were blocked with tetrodotoxin (10^{-5} g/ml) or suppressed by $Tris^+$ substitution, and the Ca^{2+} currents were blocked with Ni^{2+}, Co^{2+}, or Verapamil (hydrochloride of α-isopropyl-α[(N-methyl-N-homoveratryl)-γ-aminopropyl]-3,4-dimethoxy-phenylacetonitrile, Knoll Pharmaceutical Co., Whippany, N. J.) in doses described in the accompanying paper (Akaike et al.). A typical leakage I-V relationship shown in Fig. 8 is linear over most of its range, but rises more steeply at very large depolarizations. Substitution of isethionate⁻ or methane-
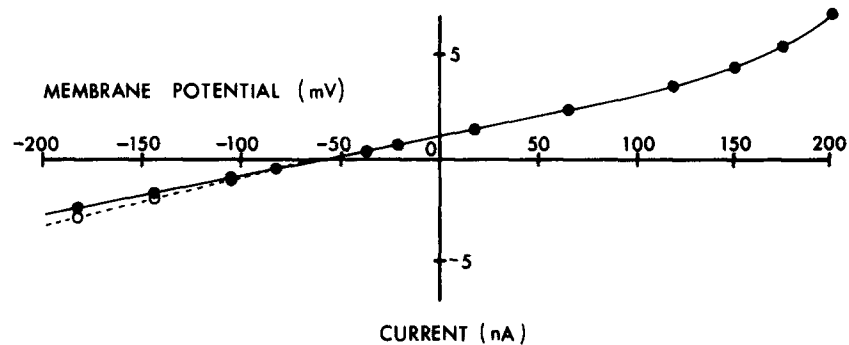

FIGURE 8. Evaluation of the leakage resistance, R_l. Current-voltage plot of the leakage current after blockage of active currents by internal perfusion with Cs aspartate (Table I), extracellular perfusion with Tris-Cs solution (Table I), and 10^{-5} M Verapamil. The shunt currents have not been nullified. The solid line is without correction for the shunt resistance and $R_{sh}:R_m$ was 10:1. Values for R_l obtained with intracellular micropipettes are shown by the dashed line.

sulfonate⁻ for internal aspartate⁻ or external Cl⁻ had no appreciable effects on the leakage I-V curves.

Voltage Clamp Currents and Their Separation in Internally-Perfused Neurons

Neurons were not classified according to appearance, position, or spontaneous activity, and some variability in results was encountered due to differences among neurons.

At small depolarizations from the holding potential of −60 mV, an inward current arises which inactivates very slowly (Fig. 9 A). An inward tail current is also observed but is not shown in this record. At more depolarizing voltage steps an inward transient appears and is followed by an outward current which, at its maximum, is usually about four-to-five times larger than the peak of the transient inward current (Fig. 9 A). The tail currents at these larger depolarizing steps are outwards. A transient outward current similar to that reported by Neher (1971) and Connors and Stevens (1971) was activated by hyperpolarizing prepulses. Both outward currents are suppressed by internal perfusion with K-

substituted Cs aspartate. Cs^+ is also substituted for external K^+, and the net result of these twin substitutions is that the resting membrane potential is only changed slightly. Thus, the change from K aspartate to Cs aspartate is accompanied by a depolarization of 10–20 mV from the resting potential, and when Cs^+ is substituted for K_o^+, the membrane repolarizes by 10–20 mV.

The time-course of current blockage is shown in Fig. 10. The outward currents are reduced and finally abolished whereas the inward currents become larger. A persistent inward current is now apparent at +25 and +40 mV, as well as −38 mV, although it is not obvious at +115 mV even after correction for leakage current (Fig. 9 B). The tail currents (not shown) are inwards at all depolarizing voltages. The time-course of the effects of these ionic substitutions is the same as the mixing times shown in Fig. 4. The outward currents were not
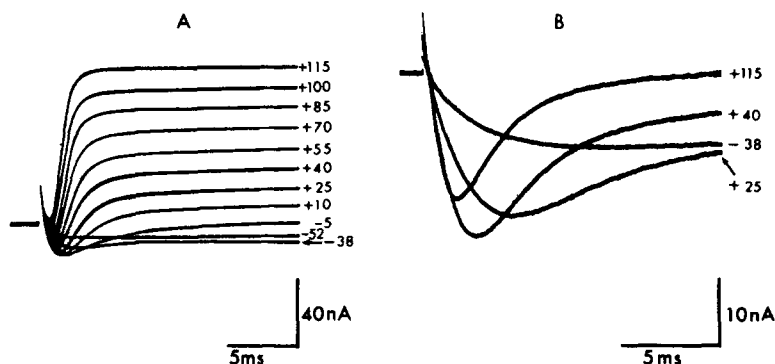

Figure 9. Effects of internal perfusion with Cs aspartate on voltage clamp currents. (A) Control clamp currents elicited by voltage steps indicated by the numbers. V_h = −60 mV. Intracellular perfusion was with K^+ aspartate (Table I). (B) Clamp currents 15 min after changing to Cs aspartate. Current is amplified 4 times greater than in A. The outward current is blocked and the inward current increased.

reduced further by substituting 50 mM TEA^+ for extracellular Na^+. However, when K aspartate was used in the internal perfusate, 100 mM TEA^+ extracellularly blocked the outward currents. In some experiments internal TEA^+ in amounts up to 10 mM was used, but the leakage currents became too large and the cell's resting potential and resistance deteriorated. The neurons appeared to tolerate internal Cs^+ better than extracellular TEA^+, so this was the preferred method of blocking outward currents.

The inward current could be separated into two components, a Na^+ current and a Ca^{2+} current. The Na^+ current was blocked by TTX or by equimolar substitution of $Tris^+$ for Na_o^+ and accounted for some 30% of the total inward current in a Cs^+-perfused neuron (Fig. 11 C). The TTX had to be made freshly from acidic stock solutions, because older samples in neutral solutions had no effect. When the Na^+ current was blocked with TTX, substitution with $Tris^+$ had no further effect on the remaining inward current. As already reported (Lee et al., 1977), treatment for 2–3 min with 0.1% trypsin in the extracellular

fluid selectively destroyed the TTX sensitivity without affecting the Na⁺ current and its reversal potential. The Ca²⁺ current and its blockage by Ni²⁺, Co²⁺, or Verapamil were unaffected by such doses of trypsin (Lee et al., 1977).

The remaining inward current was carried by Ca²⁺ ion (Fig. 11 B). All neurons showed a transient Ca²⁺ current and many, but not all, showed a slowly
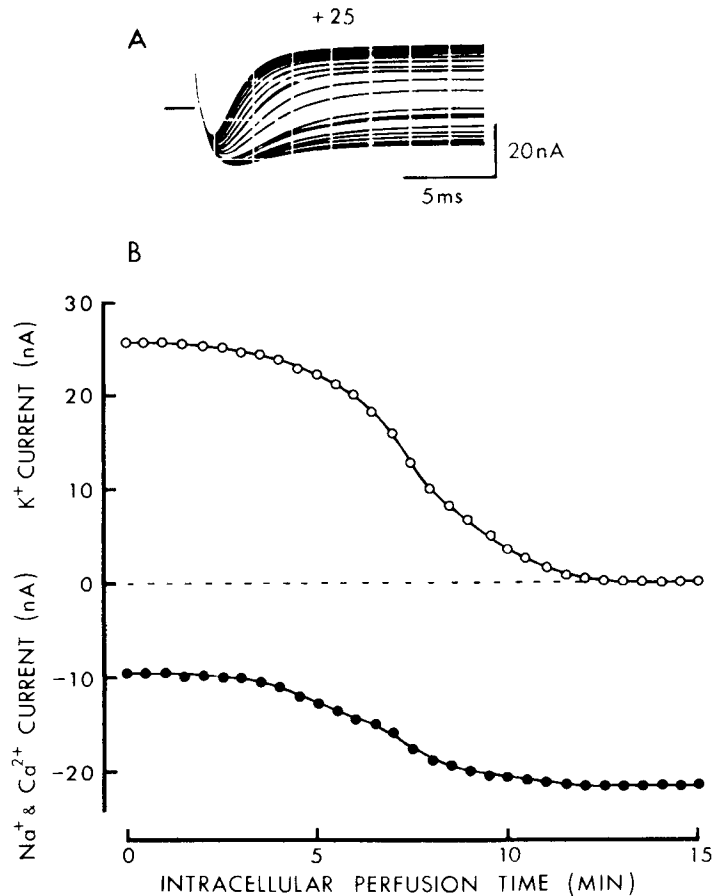

FIGURE 10. Time-course of changes in clamp currents after intracellular perfusion with Cs aspartate. (A) Currents evoked by steps from −60 to +25 mV. (B) Corresponding values for peak inward and outward currents. 0 K⁺ current occurred 13 min after Cs perfusion.

inactivating Ca²⁺ current which appeared at smaller depolarizing voltages. The Na⁺ current remaining after blockage of I_{Ca} has not been as extensively investigated, but its onset appears to be slightly faster and it appears to inactivate more rapidly (Fig. 11 C). The Ca²⁺ currents were blocked by Co²⁺ and Verapamil, although in subsequent experiments we found Ni²⁺ to be the most effective blocker. They were not affected by TTX at doses greater than those which blocked the Na⁺ current.

Effects of Intracellular Anions upon the Inward Currents

It is well known that the responsiveness and survival of internally-perfused squid axons are markedly affected by the choice of anion (Adelman et al., 1966; Baker et al., 1961; Tasaki et al., 1965). The order of anion favorability was examined, therefore, in internally-perfused snail neurons. The voltage clamp currents remained unchanged for upwards of 5 h when either aspartate⁻ or Cl⁻ was used as the internal anions, but the leakage currents were smaller with aspartate⁻. The results with F⁻ and $H_2PO_4^-$ as anions were quite different, however. After 30 min of perfusion with CsF, the inward currents were similar in magnitude and time-course to those obtained using aspartate⁻ or Cl⁻, and TTX, Verapamil, Co^{2+}, and Tris substitutions were effective blockers. After 40 min in F⁻ the inward currents and leakage currents were reduced and the clamp current capacitative transients were prolonged. At still longer perfusion times the transient peak inward current was abolished. The inward current that persisted did not inactivate and was no longer affected by TTX or Verapamil. When $H_2PO_4^-$ was used, the leakage currents were much greater than those observed using aspartate⁻ or Cl⁻, and the clamp capacitative current transient was prolonged. A selective suppression of I_{Na}, as reported by Kostyuk et al. (1975), was not observed.

DISCUSSION

Method

The suction pipette method gives values for resting membrane potential, action potential, and membrane resistance that are comparable to those obtained using the customary method, namely, intracellular micropipettes. The obvious advantages of the suction pipette method are (a) the capability to change the intracellular ionic composition, and (b) the enhanced response time of the voltage clamp because disruption of the plasma membrane by the platinized Pt wire provides a low resistance pathway to the cell's interior. Voltage clamp of nerve cell bodies with micropipettes is much slower and resolution of the early currents is not possible. As we have shown, the neuron may be avulsed from its axon and still heal over, as can cut cardiac cells. This leads to excellent spatial voltage control, and axonal contamination is avoided. It is of interest that the isolated soma does not produce all-or-none action potentials probably because the outward K⁺ current is much greater than the inward currents. Standen (1975a) reported action potentials in snail neurons in the absence of Na_o^+, but these may have been Ca^{2+}-dependent action potentials arising from the attached axons. Ca^{2+}-dependent action potentials in *Aplysia* axons have been observed by Horn (1977).

Isolation of the cell's interior from the bath is essential because the inward currents in particular are so small. The high degree of isolation achieved in the present experiments may be related to the fact that the glial covering of snail neurons examined by electron microscopy is incomplete (Kostyuk et al, 1974a).

Ionic Currents in Internally Perfused Nerve Cell Bodies

An outward current which behaves like the delayed rectifier of squid axon has

been identified (Connors and Stevens, 1971; Hodgkin and Huxley, 1952). Inasmuch as it is blocked by internal Cs^+ and external TEA^+, we attribute it to K^+. Other K^+ currents referred to in the introduction have not been separated as yet although the fast, transient current has been observed. These K^+ currents appear to be blocked by substituting Cs^+ for K^+ intra- and extracellularly. The inward currents are carried by Ca^{2+} and Na^+ ions and can be separated pharmacologically.

The Na^+ current is sensitive to TTX in larger doses than those required in squid axon. Similar results were observed in *Aplysia* (Brodwick and Junge, 1972; Geduldig and Gruener, 1970) and snail neurons (Moreton, 1968). However, we
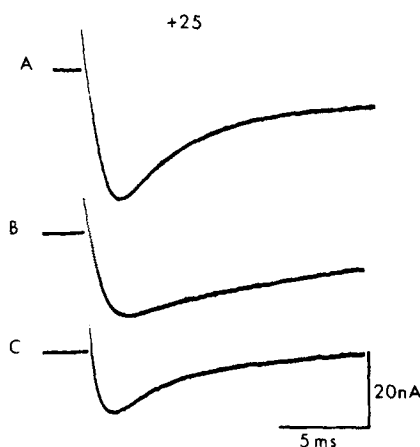

FIGURE 11. Separation of Na^+ and Ca^{2+} currents. V_h = -60 mV. Outward currents blocked by internal perfusion with Cs aspartate. External solution is Na-Cs Ringer. (A) Before separation. (B) In TTX 5 × 10^{-6} M. The inward current at +25 mV is reduced. Similar results are obtained using Tris-Cs Ringer. Pretreatment of the neuron with trypsin (0.1% for 1-2 min) prevents the TTX action but does not affect the Tris action. (C) After removal of TTX and 30 min after substitution of Co^{2+} for Ca^{2+} in the extracellular solution. The remaining inward current is blocked by TTX or Tris. Pretreatment with trypsin prevents the action of TTX but not that of Tris. The onset of the Na^+ current is faster in this neuron.

have found that it is essential to use recently prepared TTX. The TTX sensitivity is abolished by external trypsin without affecting the Na^+ current (Lee et al., 1977). This explains the absence of TTX sensitivity in dispersed neurons pretreated with trypsin (Kostyuk et al., 1974a) and points out another important advantage of the suction pipette method, namely, that enzymatic isolation of single neurons is not required.

The leakage current has been identified as the current that remains after blocking the K^+, Na^+, and Ca^{2+} voltage-dependent currents. Under these circumstances, part of it appears to be similar to the leakage currents recorded by small hyperpolarizing pulses around the resting potential of the untreated neuron. Therefore, it may not be greatly affected by the treatments used to block the active currents. It does not appear to be markedly time-dependent but it is clearly voltage-dependent, increasing at large depolarizing voltages. As we

have noted, this becomes important in the evaluation of inward currents at these voltages. The rectification may be due to Cs^+ transported through K^+ conductances.

Action of Internal Anions on the Inward Currents

The voltage-dependent inward currents and the leakage currents are greatly affected by the choice of anions used in the intracellular perfusate. The order of favorability is aspartate$^-$ $\geq$ Cl^- $\gg$ $H_2PO_4^-$ > F^-. The sequence is an inversion of the lyotropic series. The choice of anions used in the internal perfusate of squid giant axons (Adelman et al., 1966; Baker et al., 1961; Tasaki et al., 1965) and barnacle muscle fibers (Tasaki, 1968) may also affect their electrical properties markedly. However, the order of favorability in squid giant axon is F^- > $H_2PO_4^-$ > aspartate$^-$ > SO_4^{2-} $\geq$ Cl^- > Br^- which follows the lyotropic series. In barnacle the sequence is acetate$^-$ > isethionate$^-$ > aspartate$^-$ > F^- > $H_2PO_4^-$ > SO_4^{2-} > Cl^- > I^- which is neither the lyotropic series nor an arrangement based on valence, size, or polarizability.

We cannot explain the prolongation of the current transient when F^- and $H_2PO_4^-$ were used. Nor can we account for the persistent inward current at long times. Polarization of the current electrode seems unlikely in view of the small currents, and the results were present when a chlorided Ag wire was substituted. Some change in series or shunt resistance could be responsible.

Our results differ from those reported by Kostyuk et al. (1975). They found that intracellular Cl^- had deleterious effects on the neuron's survival. Survival was enhanced by using F^- as the intracellular anion, and this also separated an inward Na^+ current. When $H_2PO_4^-$ was used, an inward Ca^{2+} current was separated. None of these observations were corroborated in the present experiments. However, they used a different method to change intracellular composition. In their experiments the plasma membrane of part of the *Helix pomatia* neuron was made permeable by external perfusion with a calcium-free solution so that direct access to the cytoplasm did not occur, as is the case with our suction pipette method. In a report published after we had submitted the present papers, these authors now rupture one of the membranes but still find F^- and $H_2PO_4^-$ the preferred anions (Kostyuk and Krishtal, 1977).

The differences between results may be due to other differences between the methods or to species differences. It is not due to pretreatment with trypsin, which was used in their dialysis experiments, because this enzyme did not affect the anionic sequence in our experiments. More recently these authors have reported that F^- suppresses the Ca^{2+} conductance by destroying an associated asymmetry current (Kostyuk et al., 1977). The effect is associated with a small persistent inward current not very different from that which we have observed so that the differences between our results may not be as great as their earlier reports suggest (Kostyuk et al., 1975, 1977).

As we have noted, the electrical properties of molluscan nerve cell bodies are much more varied than those of molluscan axons. In the same cell, the soma has Ca^{2+} and Na^+ inward currents, a variety of K^+ conductances, and a host of receptor sites for potential neurotransmitters. Moreover, the specific resistance of the nerve cell bodies from which the squid giant axon arises is considerably

greater than that of the axon (Carpenter, 1973). The striking differences between preferred anion sequences is further evidence for the different natures of these two excitable membranes.

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