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DISTRIBUTION OF Na^+ AND K^+ CURRENTS IN SOMA, AXONS AND GROWTH CONES OF LEECH RETZIUS NEURONES IN CULTURE

BY U. GARCÍA*, S. GRUMBACHER-REINERT, R. BOOKMAN‡ AND H. REUTER†

Department of Pharmacology, Biocenter, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

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Summary

1. Leech Retzius neurones were isolated by a new technique which allowed investigation of macroscopic currents over the surface of the cell body and the axons using loose patch-clamp. The distribution of ion current densities was measured for neurones that had just been removed from the CNS, and for cultured cells in which neurite outgrowth had begun. To standardize the mapping procedure, the same patch electrode was used at various sites along the neurone.

2. Immediately after isolation of the cell, rapidly activating and inactivating Na^+ currents were recorded from distal segments of the axons, but not from the soma or the proximal segment. Na^+ currents were isolated by using patch electrodes containing tetraethylammonium (TEA^+) and 4-aminopyridine (4-AP) to block K^+ channels and Cd^{2+} to block calcium channels. Na^+ currents in all regions of the neurone where they could be recorded were similar in their voltage dependence and kinetics. The Na^+ current density was highest at the broken tips of the axon stumps.

3. Neurites began to extend from the broken axon tips approximately 30 min after isolation. Newly grown processes showed a high density of Na^+ currents at their growth cones. After 2 days in culture the current densities became more uniformly distributed and Na^+ currents could then be recorded in the soma and proximal axon segments.

4. In agreement with earlier studies made with conventional two-electrode voltage-clamp, three principal K^+ currents were detected in Retzius cells: a rapidly activating and inactivating A-type current blocked by 4-AP (I_A); a more

* Present address: Department of Physiology, Ctr. de Invest. y de Estudios, Avanzados del IPN, Apa 14-740, Mexico, DF.

‡ Present address: Howard Hughes Medical Institute, Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104, USA.

† Present address: Universität Bern, Pharmakologisches Institut, Friedbühlstrasse 49, 3010 Bern, Switzerland.

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slowly activating and inactivating delayed K^+ current blocked by TEA^+ (I_{K1}); and a Ca^{2+}-activated K^+ current (I_C). Immediately after isolation of the Retzius cell, both rapid A-type and slow delayed K^+ currents were distributed more uniformly than Na^+ currents over the soma and axons. In their voltage sensitivities and kinetics, these two K^+ currents were markedly different from each other; their characteristics were, however, constant in different regions of the cell.

5. Ca^{2+} currents were too small to be measured directly during depolarizing pulses. However, tail currents were large enough to demonstrate the presence of Ca^{2+} channels in the proximal segment of the axon and in the soma; the currents were not sufficiently large to resolve their spatial distribution.

6. It is concluded that ion channels are present in newly grown membranes and that the density of Na^+ channels is highest in the tips of distal axon stumps from which outgrowth begins. By contrast, K^+ currents are distributed more uniformly along the neurone.

Introduction

For a neurone to receive, integrate and transmit signals effectively, different populations of ion channels must be distributed over its surface membrane in a functionally appropriate pattern. For example, in many neurones the relative proportions of voltage-sensitive Na^+, Ca^{2+} and K^+ channels are different in different parts of the cell such as cell body, axon, dendrites, nodes of Ranvier, internodes and presynaptic terminals. Moreover, the pattern of distribution is characteristically different for each type of neurone (Waxman and Ritchie, 1985; Angelides *et al.* 1988; Thompson and Coombs, 1988; Byerly and Leung, 1988). Identified leech neurones such as Retzius and sensory cells display highly distinctive electrical properties and action potentials. By two-electrode voltage-clamp it has been shown that these differences can be explained in terms of the distribution and properties of K^+ channels (Stewart *et al.* 1989*b*).

This study describes experiments with two main aims: first, to compare the distribution of ion channel densities in various sites on the Retzius cell of the leech immediately after its removal from the CNS; second, to follow how the distribution changes as growth of new neurite processes occurs in culture. The Retzius cells are unipolar, serotonergic neurones which have a modulatory role in the animal and play a part in the swimming behaviour. In culture the cell sprouts rapidly on appropriate substrata and reliably forms specific chemical and electrical connections with an appropriate target cell (Nicholls, 1987) such as the pressure-sensitive P cell. By a novel technique we can now remove the cell body together with the proximal axonal segment and the primary and secondary axons. Detailed information is available from voltage-clamp measurements about the properties of voltage-sensitive ionic currents in cultured Retzius cells. Thus, Na^+ currents, as expected, activate and inactivate rapidly with depolarization, whereas Ca^{2+} currents activate and inactivate more slowly. At least four distinct types of K^+ currents have been identified: (a) rapidly activating and inactivating (I_A) channels blocked by 4-AP; (b) more slowly activating and inactivating K^+ channels blocked

by TEA^+ (I_{K1}); (c) Ca^{2+}-activated K^+ channels (I_C), and (d) slow, delayed K^+ channels (I_{K2}) (Stewart *et al.* 1989*b*).

There are various ways to explore the distribution of ion channels over the surface of an isolated cell. A successful approach to the measurement of Ca^{2+} channel distribution has been the use of the Ca^{2+}-sensitive indicator dye Arsenazo III to measure Ca^{2+} entry into different regions of the cell as it grows on various substrata (Ross *et al.* 1987, 1988). In spite of low spatial resolution, it has been possible to show that Ca^{2+} entry evoked by impulses is highly non-uniform, being largest in the proximal axonal segment. A completely different approach, the loose-patch method introduced by Almers *et al.* (1983), offers certain advantages over the dye method. A small area of membrane is rapidly and effectively voltage-clamped through a pipette that can be moved many times from one place on the neurone surface to another without damaging the cell. With appropriate fluids in the pipette one can selectively isolate currents carried through Na^+, Ca^{2+} and specific types of K^+ channels.

The following questions have been studied with this method. (1) How are voltage-sensitive Na^+ channels distributed over the surface of a Retzius cell immediately after removal; are there specific regions of high channel density? (2) Are the voltage sensitivity and kinetics of Na^+ currents similar in different areas of the neurone? (3) Are Na^+ channels redistributed as new neurites extend? (4) Are the various types of K^+ channels distributed in the same manner as Na^+ channels, and are they distributed separately or together? (5) Can Ca^{2+} channel distribution be measured by this method? Brief accounts of some of these experiments have been reported elsewhere (Bookman *et al.* 1987; Garcia *et al.* 1989; Nicholls and Garcia, 1989).

Materials and methods

Preparation

Ganglia were dissected out of the leech, pinned in a Petri dish and the capsules opened as described previously (Dietzel *et al.* 1986). They were then gently shaken during mild enzyme treatment with collagenase–dispase ($2\,mg\,ml^{-1}$) for 30 min. Individual Retzius cells were identified in leech ganglia by direct observation of their location. A new procedure has been devised to isolate the cell body together with long processes. First, all the small cells surrounding the Retzius cells in the central packet were sucked out one by one; the ganglia were re-incubated on a shaker with enzyme for a further 30 min. At this time the Retzius cell could be gently sucked out together with a length of axon attached to the initial segment or 'stump'. The axon often continued beyond the first bifurcation point, providing a cell with primary and secondary processes (Fig. 1). Cells were immediately plated in normal L-15 medium on a substratum consisting of Concanavalin A, as previously described (Chiquet and Acklin, 1986). Video recordings during the first 24 h showed that the initial segment or stump contracted and became thicker.

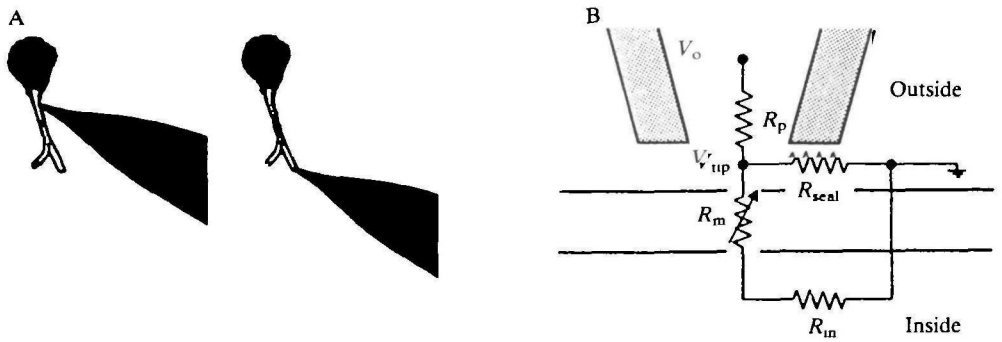

Fig. 1. (A) Photograph of a Retzius cell with a loose-patch electrode 1 h after removal from a leech ganglion. Seals can be formed at different sites such as the cell body, the axon hillock ('stump', left) and the secondary processes at its distal tip (right). (B) Simplified diagram of the equivalent circuit for a loose-patch pipette in contact with neuronal membrane. R_p, pipette resistance; R_m, membrane resistance. R_s, seal resistance. R_{in}, internal resistance; V_o, command potential. V_{tip}, potential at the tip of the pipette (after Stühmer *et al.* 1983).

Pipette and bath solutions

Normal L-15 medium consisted of Leibovitz L-15 medium (GIBCO) supplemented with 2 mmol l^{-1} glutamine (GIBCO), 6 mg ml^{-1} glucose, 0.1 mg ml^{-1} gentamicin sulphate (Garamycin; Schering, Kenilworth, NJ) and 2 % foetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel).

Solution I, for recording Na^+ currents, contained (mmol l^{-1}): TEACl, 25; $CdCl_2$, 2; 4-AP, 5, in normal medium.

Solution II, for recording Na^+ currents, contained (mmol l^{-1}): TEACl, 25; $CdCl_2$, 2; 4-AP, 5; NaCl, 125.

Solution III, for recording transient K^+ currents, contained (mmol l^{-1}): TEACl, 25; KCl, 4; $CdCl_2$, 2.

Solution IV, for recording delayed K^+ currents, contained (mmol l^{-1}): 4-AP, 5; $CdCl_2$, 2; KCl, 4.

Solution V, for recording Ca^{2+} currents, contained (mmol l^{-1}): TEACl, 25; 4-AP, 5; $CaCl_2$, 10; KCl, 4.

Solution VI, for recording Ba^{2+} currents, contained (mmol l^{-1}): $BaCl_2$, 75; TEACl, 50; Hepes, 20. This solution was used in the pipette and the bath.

Solutions II–V were buffered to pH 7.4 with TrisCl. The osmolarity was adjusted to 350–370 mosmol l^{-1} with *N*-methyl-D-glucamine chloride.

Techniques for loose-patch recording

Loose-patch electrodes with tip diameters of approximately 3–10 μm were pulled on a vertical puller and fire polished. Sylgard coating up to the tip considerably reduced capacity artefacts. Ag–AgCl wires were used in the pipette and in the agar bridge to the bath. The fluid in the patch pipette consisted of normal L-15 medium for the measurement of total currents. For isolation of individual ion currents, appropriate blocking agents were introduced into the

pipette. To isolate Ca^{2+} currents TEACl was injected into the cells through an intracellular microelectrode (see below).

Suitable pipettes had resistances of 0.4–0.8 MΩ. The same pipette was used for a whole series of experiments. To form the seal, the pipette was brought close to the membrane with the micromanipulator. Simultaneously, gentle suction was applied while some of the pressure of the pipette against the membrane was released. For tight and stable seals it was necessary to wait for 3 min before recording. With adequate seals, the seal resistance (R_s) was approximately 5–10 MΩ or 10 times the pipette resistance (R_p). In all experiments described below pipette and seal resistances remained constant in any one series of recordings and from patch to patch on the same cell. For results to be compared, $R_s/(R_s+R_p)$ had to be constant. Pipettes were replaced several times at the same place with a precision of 10 μm. Results obtained at any one site were repeatable after a series of experiments at other locations. The equivalent circuit (Stühmer *et al.* 1983) and photographs of the pipette and cells are shown in Fig. 1. The electronics were constructed for us by Dr W. B. Adams. The amplifier was an EPC-7 (List Electronic, Darmstadt, FRG) and the head stage, which was specially designed, contained a single LF 356. Controls for electrode and seal resistance compensation were contained in a separate box.

The technical limitations of the loose patch-clamp method have been analysed and described by Stühmer *et al.* (1983). They arise mainly from low electrical seal resistances between the pipette rim and the cell membrane. Voltage control may be poor underneath the rim, and the noise generated by the low seal resistance precludes accurate recordings of small currents such as Ca^{2+} currents. To assess voltage control, in some experiments we placed an intracellular microelectrode as close as possible to the extracellular patch electrode. In an extreme case we found that during a voltage pulse of 80 mV generating a 4 nA outward current, the voltage error at the end of the 20 ms pulse was 5 mV. There was no noticeable voltage escape at the beginning of the pulse during the flow of a 2 nA inward current. Since most currents recorded in our study were only about half these sizes, inadequate voltage control should not have been a major source of error. Nevertheless, current densities varied greatly from one cell to another. Therefore, for statistical analyses, relative rather than absolute comparisons have been made. A satisfactory aspect of these experiments was the reproducibility of currents measured at the same spot time after time, after the pipette had been removed and the pipette fluids changed.

Holding potentials and pulses were applied directly by the pipette. Most records show displacements from an unknown resting potential, which, when measured by an intracellular microelectrode, was between −30 and −55 mV. A membrane potential of −40 mV was assumed for all plots of current–voltage relationships. One problem with loose-patch recording is that large currents flow through the clamped area of membrane and can alter the resting potential. As shown previously (Bookman *et al.* 1987) this was not a serious problem provided that pulses lasting less than 50 ms were used (see Discussion). With longer pulses,

outward K^+ currents and leak currents became sufficiently large to alter the membrane potential of the cell as a whole, as measured by the intracellular microelectrode. Such intracellular measurements were made in the soma and primary axons but were not feasible in growth cones.

Pipette solutions were changed by ejecting them with positive pressure applied to the rear end of the pipette and sucking them up by applying negative pressure. In experiments using solutions I–V in the pipette, the bath solution was normal L-15 medium. All experiments were carried out at room temperature.

Data analysis and leak subtraction

In all experiments the depolarizing pulse was followed by three pulses in the opposite direction scaled to one-third amplitude for subtraction of leakage and capacitative currents. The currents were digitalized and stored on a laboratory computer (HNC/DECLAB 23).

TEA^+ injection

Ca^{2+} currents were recorded after injecting TEACl into the cell or into the stump to block K^+ channels more efficiently. Constant depolarizing current (1–2 nA) was passed through the TEA^+ microelectrode for 15 min. The loose-patch pipette was filled with solution V containing a high Ca^{2+} concentration. The effect of intracellular TEA^+ on K^+ currents could only be observed near the site of injection. To record K^+ currents, Na^+ currents were reduced by using Na^+-free solutions in the pipettes. Tetrodotoxin and saxitoxin (alas!) do not block leech sodium channels.

Results

Isolation of Na^+, K^+ and Ca^{2+} currents by loose patch

Characteristic inward and outward membrane currents were evoked by applying depolarizing pulses to the surface of a Retzius cell minutes after it had been removed from the CNS of the leech. Fig. 2A shows records of total currents at the tip of the secondary process obtained with normal L-15 solution in the bathing fluid and in the pipette. Separation of inward and outward currents was achieved by altering the pipette solution.

To reveal Na^+ currents in isolation, pipettes were filled with solutions containing TEA^+, 4-AP and $CdCl_2$ (solution I or II see Materials and methods), and depolarizing pulses were applied (Fig. 2B). Inward currents were maximal at approximately 0 mV (see Fig. 5) and became changed to outward ones when fluid containing 0 mmol l^{-1} Na^+ was present in the pipette.

A steady 20 mV depolarization of the patch membrane by current applied through the pipette inactivated the inward current observed during a depolarizing step. Together, these results revealed the presence of voltage-sensitive, rapidly activating and inactivating Na^+ channels in the patch. The properties of these Na^+ channels resemble those observed by two-electrode voltage-clamp, with the

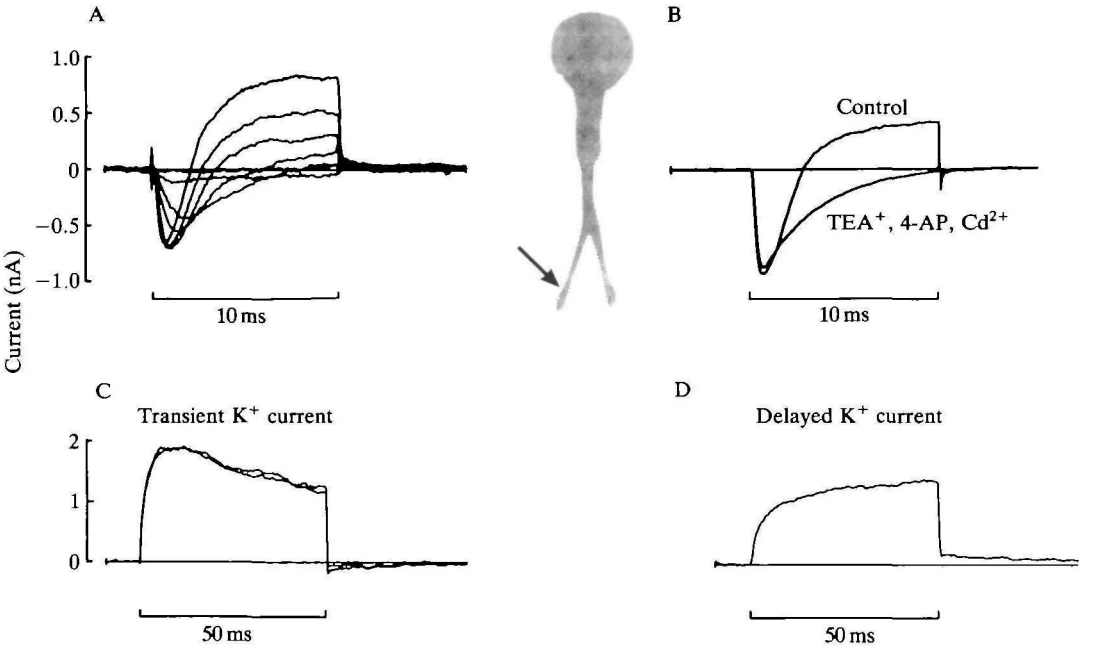

Fig. 2. (A) Total current at the tip of a secondary process recorded with a pipette filled with normal L-15 medium. The holding potential was −20 mV with respect to the resting potential (assumed to be −40 mV). Pulses of 12.5, 25, 37.5, 50, 62.5, 75 and 87.5 mV were applied. The records show that control of voltage and time resolution are adequate for resolving kinetics of inward and outward currents. (B) Blockage of outward current recorded by a loose-patch pipette filled with solution I containing TEA^+ and 4-AP. The blockage was reversible (see below, C and D). The control trace was recorded when the pipette contained normal L-15 medium. The holding potential was −20 mV with respect to the resting potential. 10 ms pulses of 56 mV were applied. (C,D) Transient outward K^+ current recorded with a loose-patch pipette placed at the tip of a primary axon. The pipette contained TEA^+ (solution III). When the pipette solution contained 4-AP (solution IV), delayed outward K^+ current was revealed (D). The second superimposed trace in C shows the transient outward current recorded once again from the same spot with solution III (TEA^+) in the pipette. A holding potential of −20 mV was applied to the membrane and depolarizing pulses of 100 mV (C) and 69 mV (D) were given.

advantage that the rising and falling phases could be observed more accurately owing to the faster speed of the clamp and the smaller amplitude of the capacitative artefacts. The results, in addition, show that the fluid in the pipette rather than that in the bathing solution effectively determines the pattern of ionic currents flowing through the membrane patch. This is shown by the blockage of individual current components by pharmacological agents and by alterations of ionic composition.

Four types of K^+ currents have been characterized in R cells: I_A, I_{K1}, I_C and I_{K2} (Stewart *et al.* 1989*b*). Two types of K^+ currents were recorded with the loose patch in the isolated cells (Fig. 2C, D). The transient K^+ current of Fig. 2C

resembled an A current, was rapidly activating and inactivating, and was insensitive to TEA^+. The external fluid and the loose-patch pipettes contained 25 mmol l^{-1} TEACl (solution III). In Fig. 2C depolarizing pulses evoked a rapidly rising outward current that decreased during the pulse. By contrast, the K^+ current of Fig. 2D activated more slowly, did not inactivate, and was insensitive to 4-AP. In these records the loose-patch pipette contained 5 mmol l^{-1} 4-AP (solution IV) instead of TEA^+. This current resembled the I_{K1} current. No further attempt was made to separate the other K^+ current components, I_C and I_{K2}. The reliability of the method is apparent from comparison of the two traces in Fig. 2C, which were taken before and after the recording shown in Fig. 2D using the same pipette on the same spot but with different blocking fluids. The current records superimpose.

Ca^{2+} currents were considerably harder to isolate than Na^+ or K^+ currents. The difficulty arose in part from the small amplitudes of the Ca^{2+} currents and in part from the presence of a masking outward current that had activation kinetics similar to that of the Ca^{2+} current. With pipettes containing no Na^+ to eliminate Na^+ currents, as well as TEA^+ and 4-AP to block K^+ currents, only very small currents were recorded during the pulse (Fig. 3A). At the end of the pulse, however, a large inward tail current was evident. Such tail currents increased in amplitude with increasing pulse durations and potentials (Fig. 3D). With Ba^{2+} in the pipette the amplitude of the tail currents increased. Ca^{2+} tail currents were most easily observed when TEA^+ was injected intracellularly to block residual K^+ currents. Even after TEA^+ injection, Ca^{2+} tail currents were obvious mainly at or close to the site of injection. For example, after TEA^+ had been injected into the proximal axon of a Retzius cell, loose-patch recordings revealed a tail current in that region but not in the soma. After injection of TEA^+ into the soma, a Ca^{2+} tail current appeared first in the soma and then in the proximal axon after a delay of a few minutes. When Cd^{2+} was added to the pipette fluid, to block currents through Ca^{2+} channels, the inward tail was abolished and an outward current was revealed (Fig. 3B). This current had an activation half-time of the order of 1–2 ms, and very rapidly deactivated at the end of the pulse. The charge carrier for this outward current was not identified but it was found to persist in the presence of high concentrations of traditional channel blockers. Subtraction of the traces in Fig. 3B from those in Fig. 3A revealed an inward current that had been blocked by Cd^{2+} (Fig. 3C), presumably carried through Ca^{2+} channels. This 'Ca^{2+} current' had a half-time for the rising phase of 1–2 ms. The steady-state amplitude (Fig. 3D) increased with larger depolarizations, as expected. Owing to their similar amplitudes and activation kinetics, the Ca^{2+} current and the outward current effectively cancelled one another during the pulse. At the end of the pulse, however, in the absence of Cd^{2+}, a Ca^{2+} tail current was revealed that reflected the extent of Ca^{2+} current activation during the pulse. (For technical reasons relating to the subtraction procedures, the fast tail current is not visible in Fig. 3C.)

Together the results show that Na^+, Ca^{2+}, fast K^+ and slow K^+ currents can be

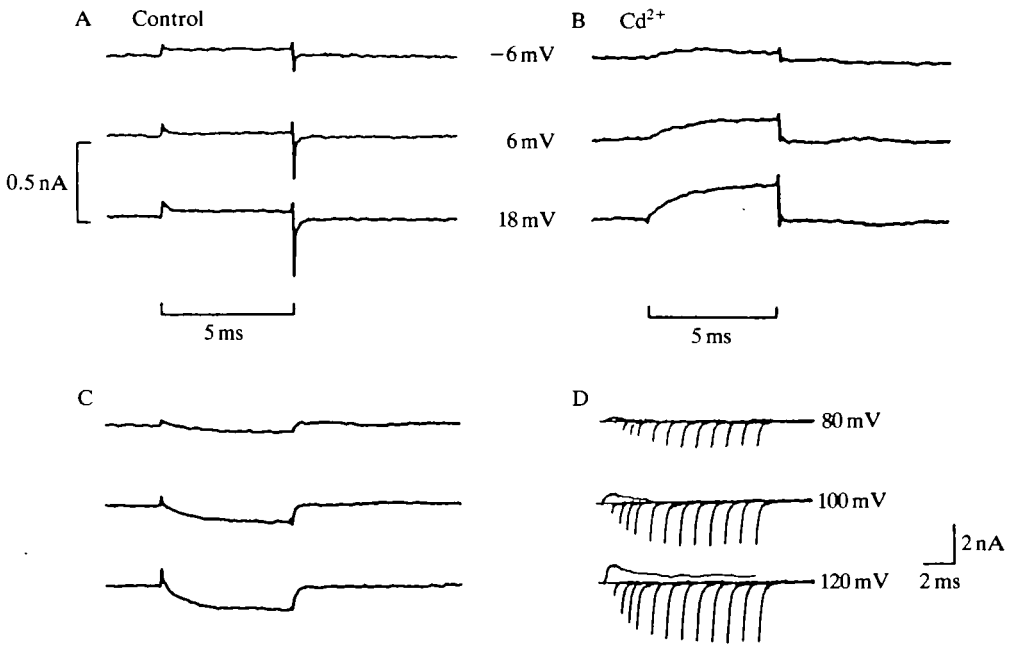

Fig. 3. (A) Ca^{2+} tail currents. 15 min after injection of 3 mol l^{-1} TEACl into the proximal axon, inward Ca^{2+} tails were recorded following depolarizing pulses applied from a pipette containing TEACl, 4-AP and 0 mmol l^{-1} Na^+ (solution V). The amplitude of the tail currents increased with larger depolarizing pulses. After injection of TEACl the resting potential measured with a microelectrode was −45 mV. No holding potentials were applied and depolarizing pulses to −6, 6 and 18 mV were given. (B) Similar pulses delivered to the same spot on the membrane by the same pipette filled with Na^+-free solution containing TEA^+, 4-AP and 1 mmol l^{-1} $CdCl_2$. An outward current was revealed that had presumably been present before but had been neutralized by an inward Ca^{2+} current. (C) Subtraction of the traces reveals an inward Ca^{2+} current (bottom) that had been masked by outward currents. (D) Amplitude of Ba^{2+} tail currents with depolarizing pulses of different amplitude and duration. Depolarizations of 80, 100 and 120 mV were applied. Both the bath and the pipette solutions contained 75 mmol l^{-1} $BaCl_2$ (with 50 mmol l^{-1} TEACl; 2 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} 4-AP and 20 mmol l^{-1} Hepes–TEAOH).

isolated. Their properties resemble those observed by conventional voltage clamp (Stewart *et al.* 1989*b*). Moreover, the currents could be recorded reliably again and again from the same spot with different bathing and pipette fluids.

Spatial distribution and voltage sensitivity of Na^+, K^+ and Ca^{2+} currents

Na^+ currents

Sodium channels were distributed over the surface of freshly dissected Retzius cells in a characteristic, non-uniform manner, as shown in Fig. 4A. Within a few minutes of removal from the CNS, large inward Na^+ currents were recorded with

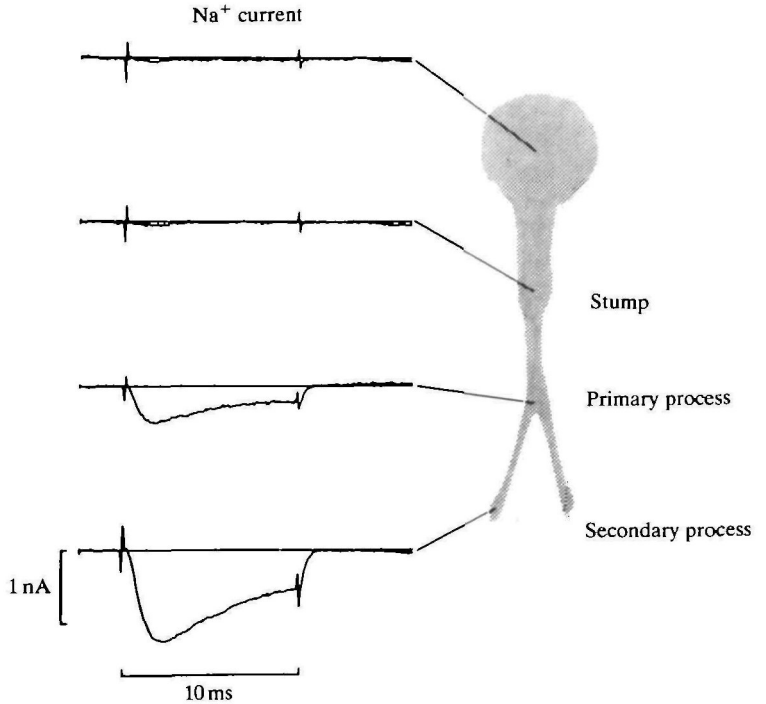

Fig. 4. Na^+ currents recorded from different sites 2 h after plating a Retzius cell on Concanavalin A. The distribution of Na^+ currents was maximal at the tip of the secondary process, decreased towards the tip of the primary axon and was negligible at the stump and the soma (from bottom to top). A holding potential of −20 mV with respect to the resting potential was applied and 10 ms pulses of +56 mV were given. The pipette contained TEA^+, 4-AP and $CdCl_2$ to block K^+ and Ca^{2+} currents (solution I). The bathing fluid was normal L-15.

step depolarizations applied to the axon. Invariably, the largest currents occurred at the broken tip of the axon from which neurite outgrowth would begin. No detectable Na^+ current could be recorded from the soma or the initial axon segment (stump) of such a cell. The occurrence of the highest channel density at the tip was correlated with damage. Retzius cells were isolated with different break-points: at the secondary axon, at the primary axon, at the end of the stump or in the middle of the stump. In each type of preparation the largest current occurred at the sealed tip. Possible reasons include membrane infolding and channel redistribution (see Discussion). The kinetics and voltage dependence of sodium channels were similar at all sites recorded. Fig. 5 shows the current–voltage relationship for records taken at the primary and at the secondary axon in a Retzius cell after 2 h in culture. At both sites the peak inward current occurred at about +2 mV. The inset of Fig. 5 shows similar activation and inactivation kinetics for the sets of channels at the two sites. The two traces recorded from the axon tip

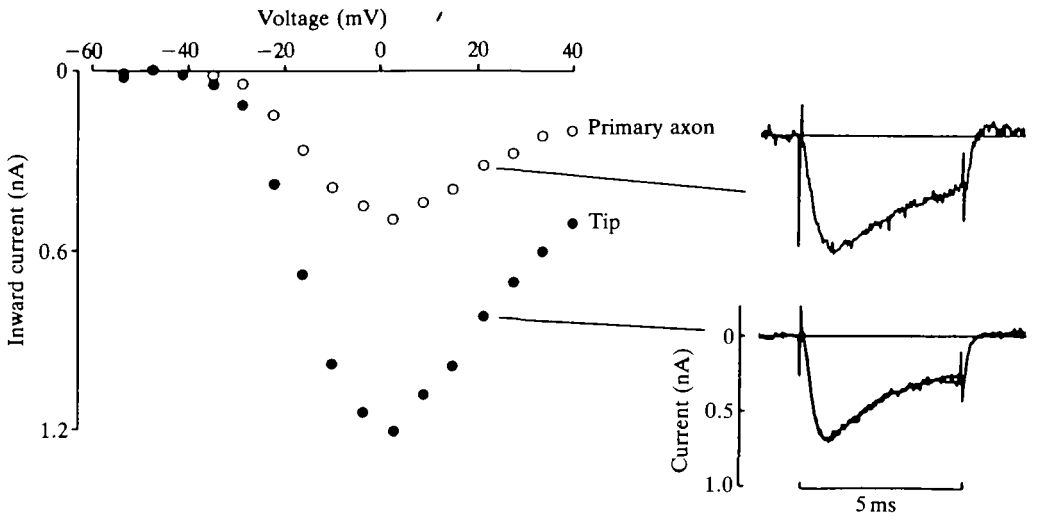

Fig. 5. Current–voltage relationships of peak Na^+ currents at two different sites on a Retzius neurone (tip of secondary axon and primary axon). The membrane potential was held at −20 mV with respect to a resting potential assumed to be −40 mV. At both sites the inward Na^+ current was activated at −30 mV and was maximal at +2 mV. The cell had been in culture for 2 h before recording. The pipette contained solution I. The insets show inward Na^+ current during pulses at the tip of the secondary processes and the primary axon. The kinetics of the Na^+ currents were similar in both places, after scaling the current at the primary axon by a factor of 2.6. The two superimposed traces from the tip were recorded before and after the recording from the axon.

in Fig. 5 were taken before and after recording from the soma with the same pipette.

Na^+ current was recorded from newly grown processes, and from growth cones, a few hours after removal. There was no obvious delay between the appearance of the membrane and the appearance of Na^+ channel activity. After 2 days in culture, Na^+ current was more uniformly distributed over the entire cell than at the time of isolation. Na^+ current could then be recorded in the soma as well as on the axons and in the growth cones (see Fig. 8).

Fast (4-AP-sensitive, I_A) and slow (TEA^+-sensitive, I_{K1}) K^+ current distribution

With a pipette containing TEA^+ solution III to block slow K^+ currents, the remaining outward transient K^+ current activated and inactivated more rapidly (Fig. 6A). As expected, it was reduced or abolished by steady depolarization of the holding potential by 20 mV. With a pipette containing 4-AP, K^+ currents were slower and did not inactivate during the 50 ms pulse (Fig. 6B).

The distribution of either current was more uniform than that of the Na^+ currents. Although variations in K^+ current density were observed in individual cells (Fig. 6), no variation was apparent when results from a number of cells were pooled (see Fig. 8). The different characteristics of fast and slow K^+ currents were

confirmed by studying current–voltage relationships (Fig. 7A). Fast and slow currents differed in their sensitivity to depolarizing steps: fast currents began to activate at depolarizing steps to −35 mV, whereas slow channels required a larger depolarization to at least −25 mV. Measurements made from the soma, initial

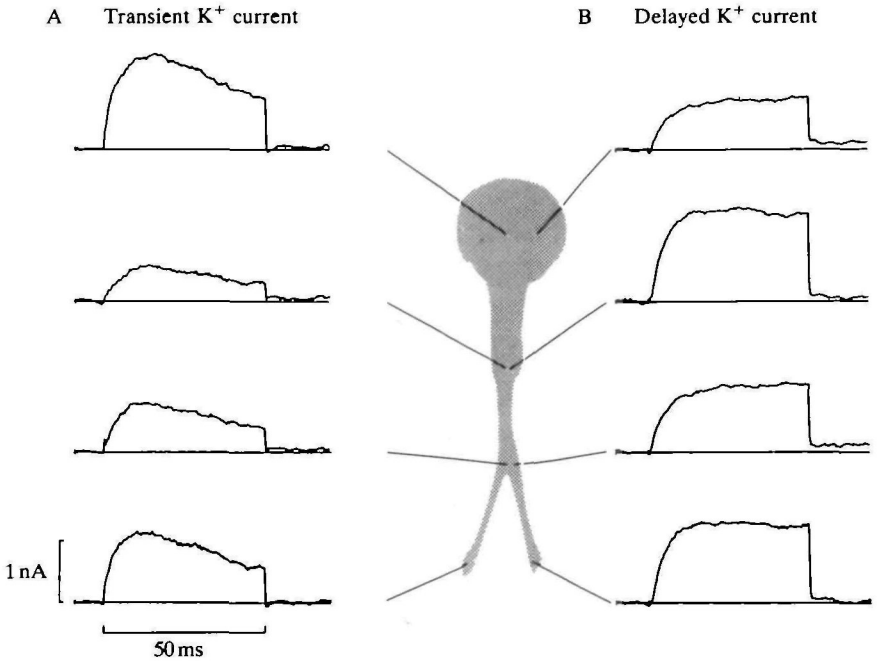

Fig. 6. Transient and delayed K^+ currents at four locations (soma, initial segment, primary axon and tip of secondary axon). (A) Transient K^+ currents recorded with pipette containing TEA^+ (solution III). Depolarizing pulses were +100 mV from a holding potential of −30 mV with respect to the resting potential. (B) Delayed K^+ current recorded with a pipette containing 4-AP (solution IV). The pulse amplitude was 70 mV from a holding potential of −20 mV with respect to the resting potential.

Fig. 7. (A) Current–voltage relationships of transient and delayed K^+ currents recorded from the same area of membrane (arrowed) of a Retzius cell growing on Concanavalin A for 2 h. The same pipette was filled with solution III for measuring delayed K^+ current (●) or solution IV for measuring transient K^+ current (○). The holding potential was −25 mV from an assumed resting potential of −40 mV. The delayed current required larger pulses for activation. (B) Current–voltage relationships of delayed peak K^+ currents recorded at two sites, the tip of the secondary axon (●) and the initial segment (○). The current–voltage relationships have the same shape; holding potential was −20 mV with respect to the membrane potential. The inset shows delayed K^+ currents recorded from the initial segment and distal tip superimposed after scaling (1.28 times, marked by arrows on graph). Note the similar kinetics. (C) Current–voltage relationships of transient peak K^+ current at the soma (●) and tip of the initial segment (○). The inset shows that the transient K^+ currents were identical after scaling (2.7 times). Holding potential was −30 mV with respect to the membrane potential.

segment, distal axons or growth cones showed no differences in the voltage sensitivity or activation characteristics: the different regions were similar in the properties of their fast or slow K^+ currents. Current–voltage relationships in the

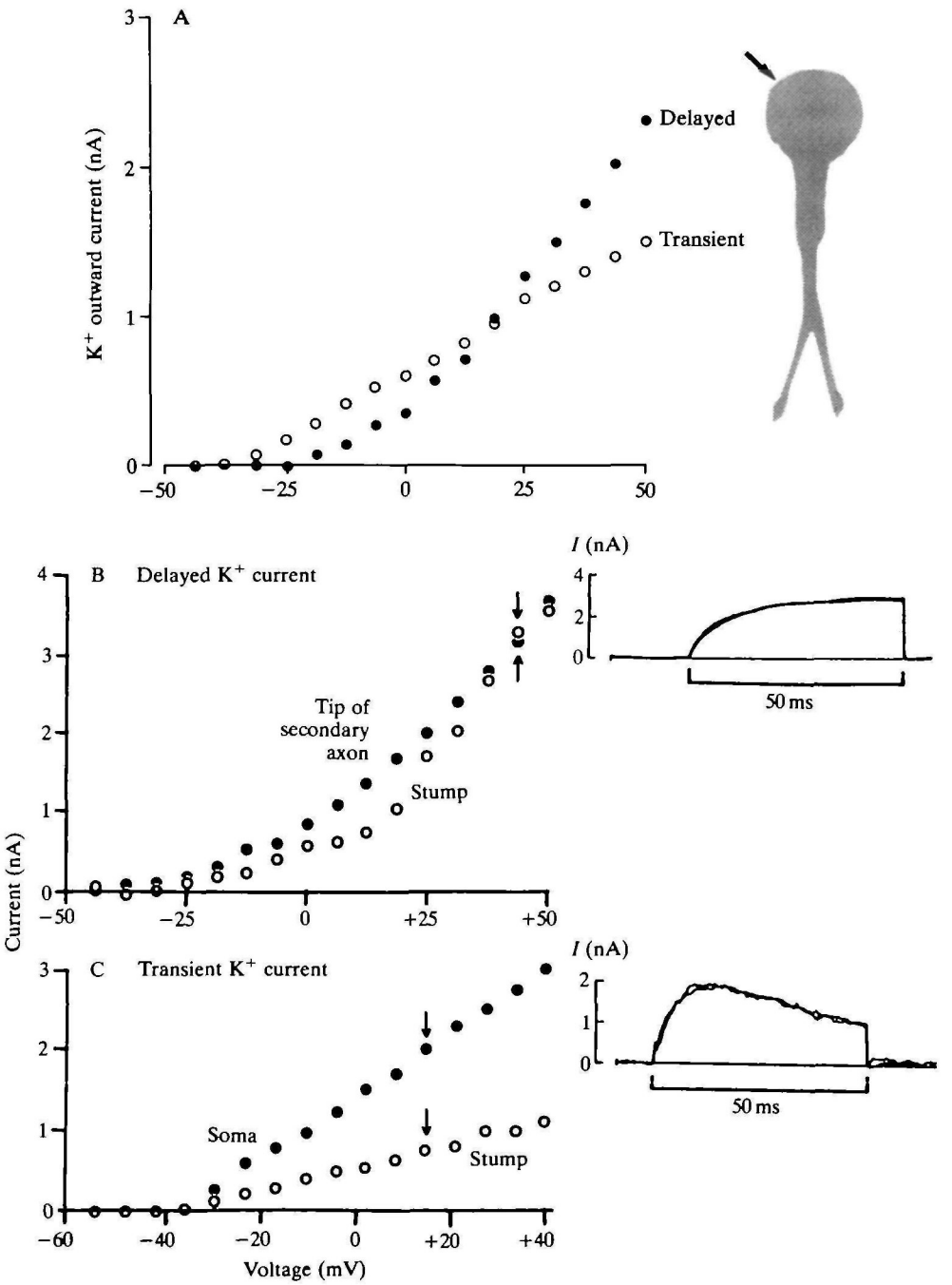

Fig. 7

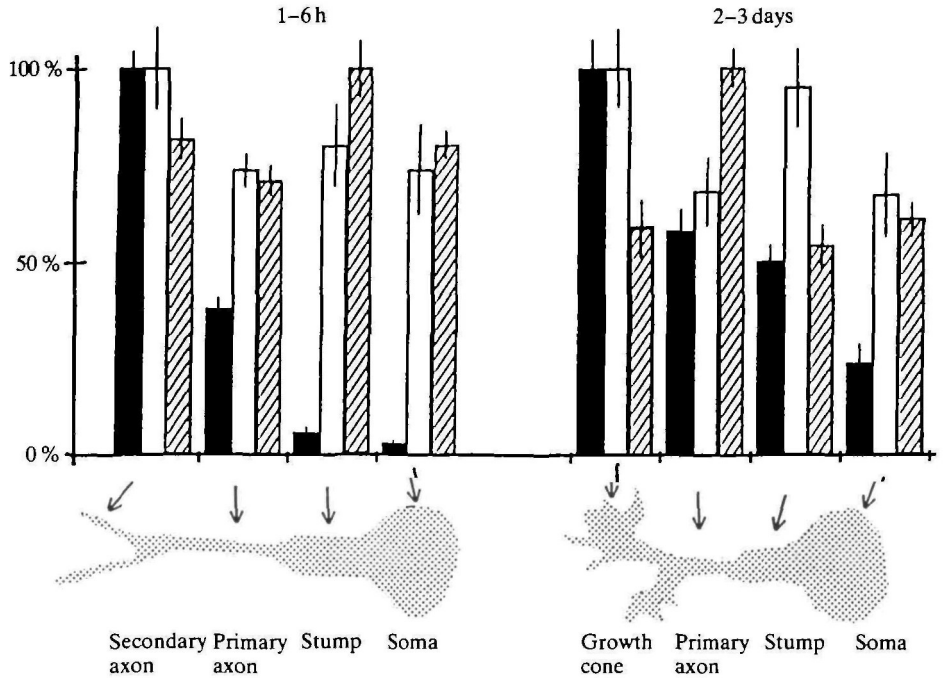

Fig. 8. The distribution of Na^+ (■), transient K^+ (□) and delayed K^+ (■) currents in Retzius cells after 1–6 h (left) and after 2–3 days in culture. Peak currents were measured from current–voltage curves resembling those of Figs 5 and 7. Currents were measured (i) for Na^+ current after 700–1000 μs, (ii) for transient K^+ current after 11.7 ms and (iii) for delayed K^+ current at the end of the 50 ms pulse. Averages from five or more cells were calculated and normalized with respect to the maximal individual current components observed for each cell. Na^+ currents were maximal at the tip of the secondary axon immediately after removal and appeared in the soma 2 days later. Bars represent standard errors of the mean.

soma and the axon for delayed and transient K^+ currents are shown in Fig. 7B,C; the kinetics of K^+ currents at different sites are given in the insets of Fig. 7.

K^+ channels, as well as Na^+ channels, appeared in growing processes and growth cones (Fig. 8). Both transient and delayed K^+ currents were recorded as soon as new processes grew out (Fig. 8). In the present experiments the transient and delayed K^+ currents were studied without any further separation of the various additional K^+ current components identified by Stewart *et al.* 1989*b*. It was, however, evident that, in addition to the 4-AP-sensitive transient K^+ currents and the TEA^+-sensitive delayed K^+ currents, other K^+ currents with distinctive properties appeared in growth cones and newly grown processes after 2 days in culture. Such currents were more resistant to TEA^+ and 4-AP. They were not analysed in detail.

Calcium channel distribution

For technical reasons it was difficult to compare quantitatively the calcium currents in different regions of the cell. The currents were small and required highly standardized seal resistances (see above). We did not investigate changes during development in culture.

Discussion

Ion channels of the Retzius neurone of the leech, studied using the loose-patch technique, showed an uneven distribution over the surface of the cell that could change during development in culture. The observed properties of the channels agreed well with those found earlier using other methods (Stewart *et al.* 1989*a*,*b*; Ross *et al.* 1987, 1988).

In cells that had just been removed from the CNS, sodium current could be recorded only in the broken extremity of the axon. After 2–3 days in culture, this current was found in the soma and proximal region of the axon also. The two types of potassium current that were studied, fast I_A and slow I_{K1}, showed variation in distribution that was not consistent between cells, yielding a uniform distribution on average.

After 2 days in culture, when neurite outgrowth was extensive, Na^+ and K^+ currents could be recorded in growth cones. Current densities in these newly formed structures were similar to those recorded in the tip of the broken axon from which neurite outgrowth occurred. This indicates that the channels may first move towards the site of damage, and later diffuse into the newly formed neurites. Another possibility is insertion of new channels into these sites *via* axonal transport of the channel proteins. In rat embryonic cortical neurones and spinal cord cells Angelides *et al.* (1988) found different lateral mobility of Na^+ channels within the membrane plane in different parts of the neurone. This would be in agreement with the first hypothesis mentioned above. In contrast, unequal distribution of Na^+ channels has also been shown in skeletal muscle fibres (Beam *et al.* 1985). Na^+ channel density is highest near the site of the neuromuscular junction of these cells. Na^+ current densities vary up to threefold over short distances and the lateral mobility of the channels is undetectably small in frog skeletal muscle (Almers *et al.* 1983). Further experiments with Retzius cells will be needed to decide whether lateral diffusion or axonal transport accounts for the highly localized distribution of these channels in the present study. Other techniques will also be needed to obtain a better resolution of the distribution of Ca^{2+} channels in these cells.

What are the functional implications of the special localization of Na^+ channels in growing Retzius cells? The highest Na^+ current density could always be recorded at the tip of the cut axons after isolation of the cells. Neurite outgrowth also occurred most actively in this region. The access resistance of the narrow neurites is presumably large. For spread of excitation into the neurites to occur, a high density of Na^+ inward current is needed to overcome the access resistance. In

addition, as discussed above, Na^+ channels may diffuse from the sites of high density into the newly formed neurite membranes. Both functional implications have still to be verified. It will also be interesting to see whether and how channel distribution is affected by synapse formation.

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