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ANALYSIS OF CALCIUM CHANNEL PROPERTIES IN CULTURED LEECH RETZIUS CELLS BY INTERNAL PERFUSION, VOLTAGE-CLAMP AND SINGLE-CHANNEL RECORDING

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Summary

1. The properties of voltage-dependent calcium channels have been measured in Retzius cells isolated from the central nervous system of the leech and maintained in tissue culture.

2. Macroscopic divalent cation currents were isolated after blocking Na^+ and K^+ currents by bathing the cells with Na^+-free solutions containing TEA^+ and 4-AP, and internally perfusing them with Cs^+ and TEA^+. Depolarizing voltage-clamp pulses activated inward currents that were larger for Sr^{2+} than for Ba^{2+} or Ca^{2+}. The peak currents were observed at +15 mV for Ca^{2+} and Ba^{2+} and at +7 mV for Sr^{2+}. Divalent cation currents were blocked by Cd^{2+} and Mn^{2+} but not by dihydropyridine blockers.

3. The activation kinetics of Ba^{2+} currents was sigmoid. The inactivation was approximately 10 % at the end of a 50 ms depolarizing pulse. Decay of Sr^{2+} and Ca^{2+} currents was larger and showed two kinetic phases. Activation and inactivation of the calcium channels were not significantly influenced by the holding potential.

4. Deactivation kinetics observed during tail currents consisted of two exponential components. At a closing voltage of −60 mV, the time constant was ≈200 μs for the fast component and 1.9 ms for the slow component. Both time constants of deactivation were voltage-dependent over the range from −80 to −20 mV, and increased at more depolarized closing voltages.

5. Single-channel activity was recorded with cell-attached patches in solution containing 75 mmol l^{-1} Ba^{2+}.

6. Taken together, the results define the characteristics of a distinctive type of calcium channel in isolated Retzius cells.

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Introduction

Several types of calcium channel with distinctive properties have been identified and characterized in vertebrate and invertebrate neurones (Reuter, 1985; Byerly and Hagiwara, 1988; Tsien *et al.* 1988). These channels differ with respect to their ion selectivity, kinetics and sensitivity to toxins (Carbone and Lux, 1984*b*; Fedulova *et al.* 1985; Fox *et al.* 1987*a*,*b*; Swandulla and Armstrong, 1988). Relatively few studies have been made on the calcium channels that play a part in physiological processes such as transmitter release (Llinas *et al.* 1981; Augustine *et al.* 1987).

The aim of the present experiments was to describe divalent cation currents in cultured Retzius cells of the leech, which extend processes to establish chemical synapses. Single Retzius cells survive for days or weeks after isolation from the central nervous system (Ready and Nicholls, 1979). In culture, they maintain their membrane properties. When co-cultured with an appropriate partner they can form chemical synapses that show marked dependence on external Ca^{2+} (Fuchs *et al.* 1981, 1982; Henderson, 1983; Henderson *et al.* 1983; Dietzel *et al.* 1986; Nicholls, 1987). Moreover, optical recordings made with the Ca^{2+} indicator Arsenazo III show that Ca^{2+} entry following action potentials is influenced by the molecular composition of the substratum and is unevenly distributed over the surface of a growing Retzius cell (Ross *et al.* 1987, 1988).

Although the characteristics of Na^+ and K^+ channels have been measured in two-electrode voltage-clamp and with loose patch studies (Stewart *et al.* 1989*b*; Bookman *et al.* 1987; Nicholls and Garcia, 1989; Garcia *et al.* 1990), surprisingly little information is available about Ca^{2+} currents in leech neurones (see Stewart *et al.* 1989*a*,*b*). Certain technical difficulties arise with conventional microelectrodes. First, the high impedances of the microelectrodes required for penetration limit the speed with which the membrane potential can be displaced and clamped (approx. 1–2 ms). The capacity current artefacts obscure the initial phases of activation and deactivation, precluding analysis of rapid kinetics. A second difficulty is that the isolation of Ca^{2+} currents is difficult in the presence of unblocked, residual K^+ currents (Stewart *et al.* 1989*a*,*b*). These problems have been largely eliminated in the present experiments by replacing the current-passing microelectrode with a low-resistance 'patch'-type pipette that passes large currents to charge the membrane capacitance rapidly ($\approx$100 μs) and that permits internal perfusion. In addition, a tight-seal cell-attached patch-clamp method has been used to examine single Ca^{2+} channel activity. With these modifications it became possible to explore the following questions concerning the voltage-dependent calcium channels present in Retzius cells in culture. (1) What are the kinetics of activation, deactivation and inactivation of Ca^{2+} channels? (2) To what extent do Ca^{2+}, Ba^{2+} and Sr^{2+} permeate through the divalent cation channels? (3) Can more than one type of calcium channel be identified? (4) What are the properties of individual calcium channels? Preliminary reports of this work have been presented elsewhere (Bookman and Liu, 1986, 1987).

Materials and methods

The techniques for the identification, isolation and culture of Retzius cells from the CNS have been described in detail elsewhere (Fuchs *et al.* 1981; Dietzel *et al.* 1986). In brief, individual Retzius cells were removed after enzyme treatment of ganglia with collagenase–dispase ($2\,mg\,ml^{-1}$), and plated in microwells coated with poly-L-lysine in L-15 culture medium containing gentamycin ($100\,\mu g\,ml^{-1}$) and foetal calf serum (2%). Measurements were made at room temperature (20–22°C) in appropriate solutions (see below). The perfusion system permitted changes in the bathing fluid to be effected within 5–60 s. Cells were examined 1–15 days after removal from the ganglion. For the formation of high-resistance seals between either the current-passing electrode or the patch pipette, it was essential that the surface of the neurone should be clean of debris.

Voltage-clamp electrodes

Current-passing electrodes were double-pulled from haematocrit tubing (Clay Adams 1021), fire-polished and insulated with Sylgard (Hamill *et al.* 1981). For these electrodes, tip openings ranged from 2 to $8\,\mu m$ and had resistances of 0.5–1.5 MΩ in our standard external solution. Voltage microelectrodes were pulled from filament glass to have resistances of 10–30 MΩ when filled with $3\,mol\,l^{-1}$ KCl. Miniature Ag/AgCl pellets (E-255, In Vivo Metrics, Healdsburg, California) were used for the patch electrodes, the microelectrodes and for the reference electrode, which was connected to the bath by an agar bridge.

Hybrid voltage-clamp techniques

Frequency response was maximized and capacitative artefacts were reduced by using low-impedance patch electrodes for passing current. The intracellular fluid was perfused in experiments to isolate Ca^{2+} current from Na^+ and K^+ currents (Kostyuk and Krishtal, 1977; Lee *et al.* 1978; Hagiwara and Byerly, 1981; Byerly and Hagiwara, 1982). The current-passing pipette was first sealed onto the cell surface with a seal resistance of 1 GΩ or more. As suction ruptured the membrane, there was a concomitant decrease of the tip resistance to about 50 MΩ, representing the parallel combination of the seal and membrane resistances. The resting potential was stabilized with hyperpolarizing current and the cell was then impaled with the high-resistance voltage-recording microelectrode.

The performance of this clamp is illustrated in Fig. 1. The membrane potential reached more than 90% of its new value in about $100\,\mu s$. Capacitative currents decayed with a time constant of approximately $45\,\mu s$. These speeds were achieved by using a conventional two-electrode voltage-clamp amplifier (Almost Perfect Electronics, Basel, Switzerland). The I–V converter headstage of a patch-clamp amplifier did not charge the whole-cell capacitance sufficiently fast for rapid activation or deactivation kinetics to be measured.

Single-channel recording

Electrodes were made from hard Pyrex glass H15–10 (Jencons Scientific,

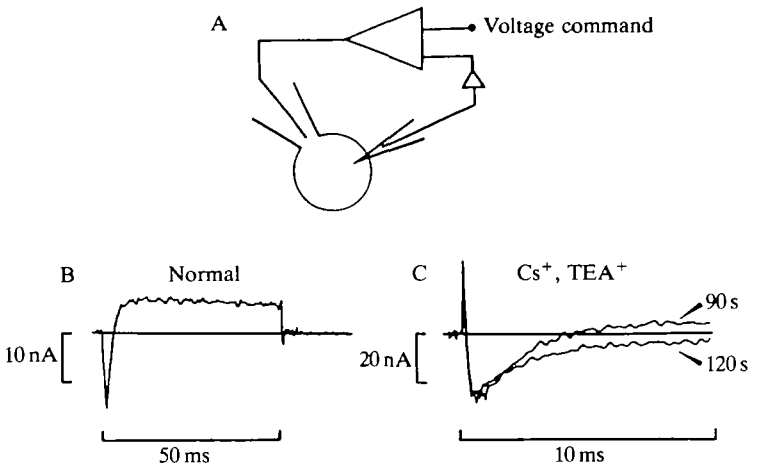

Fig. 1. Performance of internal perfusion by the hybrid voltage-clamp method. (A) Diagram of the circuit. (B) Whole-cell recording of membrane current during a 50 ms pulse from a holding potential of −60 mV to −10 mV. With 120 mmol l^{-1} K^+ in the intracellular solution and 140 mmol l^{-1} Na^+ in the external solution, inward I_{Na} was followed by outward I_K. (C) Two records of membrane current in another cell. The current-passing (perfusion) pipette contained 100 mmol l^{-1} caesium glutamate and 25 mmol l^{-1} TEA-Cl. The cell was depolarized from −60 mV to +30 mV for 10 ms. The top trace was recorded 90s after the rupture of the membrane, and the lower trace 30s later.

England) and had resistances of 2–6 MΩ. Channel activity was recorded in cell-attached patches with records filtered at 0.6–2 kHz and corrected for leak and capacity currents by adding scale-averaged records from 16 or 32 hyperpolarizing pulses with no channel activity.

Data acquisition computer and interface

The on-line data acquisition system was designed by one of us (RJB) in collaboration with C. M. Armstrong and D. R. Matteson. High-speed transfer of parallel data between the experimental interface and the computer was accomplished with a direct memory access parallel interface card (DRV11-WA, Digital Equipment Corp.) capable of transferring one 16-bit word every 2 μs. The experimental interface consisted of an optically isolated 100 kHz, 14-bit A/D converter, a 14-bit D/A converter to make voltage command pulses, D/A converters to display sweeps on an oscilloscope and associated logic to handle data transfers.

Current recording

The signal proportional to membrane current was filtered at a corner frequency appropriate for the sampling rate to prevent aliasing (LPF 902, eight-pole Bessel low-pass filter, Frequency Devices, MA, USA). The sampling rates ranged from

to 100 kHz. Linear leakage currents and capacitative currents were subtracted by the computer using the P/n method of Bezanilla and Armstrong (1977). Here, the currents recorded during four hyperpolarizing pulses scaled to one-quarter of the amplitude of the test pulse were added to the record of current from the depolarization. These hyperpolarizing 'subtraction' pulses were applied from the same holding potential as the test pulse. In this way, records of membrane current were leak- and capacity-corrected and signal-averaged (2–10 sweeps). Data acquired on-line were stored on disc.

Data analysis

For current–voltage relationships, peak measurements represent the average of five points around the peak while isochronal measurements were from a single point. Time constants and single exponential fitting of current records were obtained by first performing a logarithmic transformation of the data and then calculating a weighted linear regression. Double exponential fits were verified with an implementation of the Levenberg–Marquardt method of least-squares fitting (Wavemetrics, Lake Oswego, Oregon, USA).

Solutions

The standard internal solution contained (in mmol l^{-1}): caesium glutamate, 100; *N*-methylglucamine fluoride, 25; TEA-Cl, 25; EGTA, 2; Hepes–TEA-OH, 20. The external solution was (in mmol l^{-1}): NaCl, 140; $CaCl_2$,5; $MgCl_2$, 5; KCl, 2; Hepes–NaOH, 20. '75 $BaCl_2$' solution contained (in mmol l^{-1}): $BaCl_2$, 75; TEA-Cl, 50; $MgCl_2$, 2; 4-aminopyridine, 5; Hepes–TEA-OH, 20. Permeation of different divalent cations was tested with 25 mmol l^{-1} concentrations of Ca^{2+}, Sr^{2+} or Ba^{2+} in 50 mmol l^{-1} TEA-Cl, 20 mmol l^{-1} Hepes–TEA-OH and 75 mmol l^{-1} *N*-methylglucamine chloride. All solutions were adjusted to a final pH of 7.4 and an osmolarity of 350 mosmol l^{-1}.

Internal perfusion for isolation of currents

A serious limitation in the measurement of macroscopic calcium channel currents is contamination by currents flowing through other channels or leak pathways (Hagiwara and Byerly, 1981; Byerly and Hagiwara, 1982). With internal perfusion through the current electrode, Retzius cell bodies could be perfused with the standard internal solution containing Cs^+ and TEA^+. The exchange of intracellular fluids was complete in about 2 min. Fig. 1B shows the total current of a Retzius cell recorded during a 50 ms pulse to −10 mV while the cell was perfused with a solution containing 120 mmol l^{-1} KCl and with an external solution containing 140 mmol l^{-1} NaCl without blockers. The early inward current carried by Na^+ was followed by an outward K^+ current (compare Stewart *et al.* 1989*b*).

Fig. 1C shows recording from a different cell perfused with intracellular solution containing 100 mmol l^{-1} caesium glutamate and 25 mmol l^{-1} TEA-Cl. By the time that the upper trace in Fig. 1C was recorded (about 1.5 min after breaking in) most

of the K^+ current had already been blocked. By 2 min, net outward current had been eliminated.

Results

Single-channel currents

Single calcium channel currents were recorded from cell-attached patches in a solution containing $BaCl_2$ and TEA^+ (75 $BaCl_2$ solution). Current recorded from a patch on the initial segment or 'stump' of a Retzius cell in culture for 5 days is shown in Fig. 2. Depolarization by 30 mV caused the appearance of brief events with a duration of 2–20 ms and about 1 pA in amplitude. During 50 mV depolarizations, the currents increased in frequency and decreased in amplitude to about 0.8 pA. Channels permeable to Ba^{2+} with similar characteristics were observed in 12 patches. Detailed analyses of Ba^{2+} single-channel currents were not possible for three reasons. First, it was difficult to establish the seal resistances of 10–100 GΩ which were required for recording small single Ca^{2+} channel currents. Second, even when tight seals were established, most of the patches showed no channel activity in the presence of Ba^{2+}. The 12 successful patches were the result of literally hundreds of trials. Third, it was not possible to test decisively whether currents such as those shown in Fig. 2 were due to more than one channel in the patch (see Discussion). Accordingly, the properties of calcium channels were

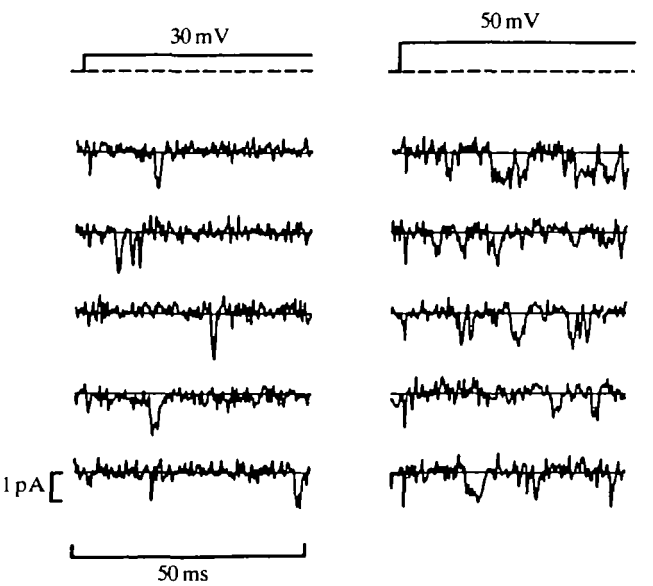

Fig. 2. Single-channel activity recorded in a cell-attached patch with 75 mmol l^{-1} Ba^{2+} in the pipette solution. The resting potential of the cell was unknown. Depolarizing command pulses of 30 mV (left) or 50 mV (right) were applied to the pipette. Leak and capacity currents were subtracted by adding scale-averaged records from 16 hyperpolarizing pulses. Records were filtered at 1 kHz.

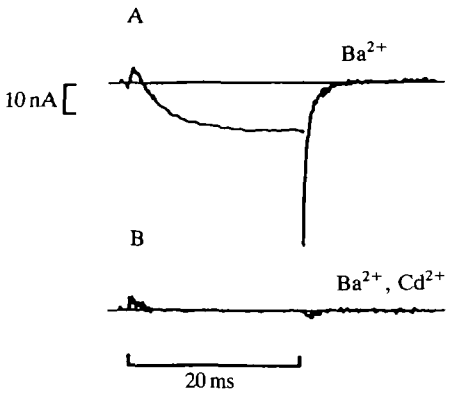

Fig. 3. Cadmium sensitivity of the Ba^{2+} current. (A) Ba^{2+} current recorded during a 20 ms pulse from −60 mV to +10 mV with 75 mmol l^{-1} Ba^{2+}, 0 mmol l^{-1} Na^+ outside and 100 mmol l^{-1} Cs^+, 25 mmol l^{-1} TEA^+ in the perfusion pipette. (B) 200 μmol l^{-1} $CdCl_2$ was added to the external solution. The slowly activating inward component and the major part of the large tail current were blocked.

studied by measuring macroscopic currents recorded with the hybrid voltage-clamp as described in the following sections.

Voltage dependence and selectivity of calcium channels

Fig. 3A shows a slowly activating inward current and a large tail current at the end of a depolarizing pulse to +10 mV from a holding potential of −60 mV, recorded in external solution containing 75 mmol l^{-1} Ba^{2+} and 0 mmol l^{-1} Na^+. Both inward and tail currents were blocked by adding 200 μmol l^{-1} $CdCl_2$ to the bathing fluid (Fig. 3B), indicating that the flow of ions was through calcium channels. A small initial transient outward current and a minor component of the tail current persisted in the presence of Cd^{2+} (see below). Dihydropyridines such as (−)202-791 or (+)202-791 (Sandoz, Switzerland; Kongsamut *et al.* 1985) at concentrations of 20 μmol l^{-1} did not block or activate Ba^{2+} currents.

The voltage dependence of Ba^{2+} currents is plotted in Fig. 4. Inward Ba^{2+} currents became apparent with depolarizations to −25 mV from a holding potential of −60 mV. With larger depolarizations up to about +15 mV the inward currents became larger. Depolarizations beyond +15 mV led to smaller isochronal currents measured at 10 ms. As the driving force diminished, the extrapolated current reversed at about +50 mV (with 75 mmol l^{-1} Ba^{2+} outside and 100 mmol l^{-1} Cs^+ inside).

The selectivity of Retzius cell calcium channels was measured with 25 mmol l^{-1} Sr^{2+} or Ba^{2+} in the bathing fluid. The current–voltage relationships of Fig. 4, all recorded from a single Retzius cell, show that Sr^{2+} currents were larger than those for Ca^{2+} or Ba^{2+}. In most experiments to be described Ba^{2+} was used as the charge carrier because it blocked K^+ channels (thereby improving the isolation of

the current) and showed less inactivation (see below) (Standen and Stanfield, 1978; Armstrong and Taylor, 1980).

The conductance–voltage (G–V) relationship for Ca^{2+} channels (Fig. 5) was calculated from the magnitude of the instantaneous current change at the end of 20 ms pulses upon return to the holding potential. This relationship is therefore a measure of those channels that were open after a depolarization of 20 ms duration. Ba^{2+} entry into the cell, estimated by integrating pulse and tail currents, showed that charge entered the cell mainly during the depolarization, with far less entry

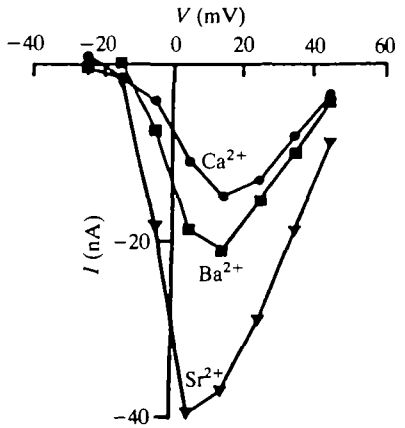

Fig. 4. Permeation of Ca^{2+} channels by Ca^{2+}, Ba^{2+} and Sr^{2+}. The concentration of divalent cations in the external solution was 25 mmol l^{-1}. The cell was superfused with the external solutions in the order Ba^{2+}, Ca^{2+}, Sr^{2+}. Current–voltage relationships were obtained by plotting isochronal currents at the end of 10 ms depolarizing pulses against membrane potential.

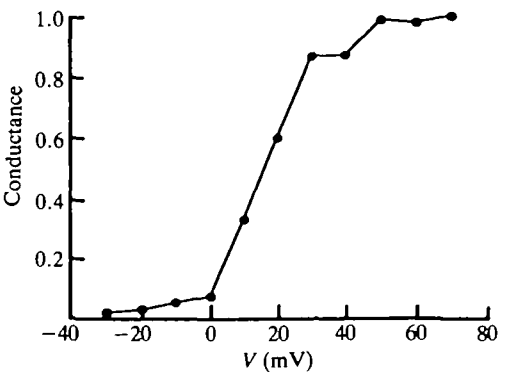

Fig. 5. Conductance–voltage relationship of the Ba^{2+} current. Conductances were calculated as $\Delta I/\Delta V$. ΔI is the difference in the current from the end of the pulse to the beginning of the tail ($I_{pulse}-I_{tail}$). The initial value of the tail current was estimated by extrapolating an exponential fitted to the tail current back to 100 μs after the end of the pulse. ΔV is the magnitude of the voltage step ($V_{pulse}-V_{holding}$). These values were normalized so that the maximum conductance was 1.0.

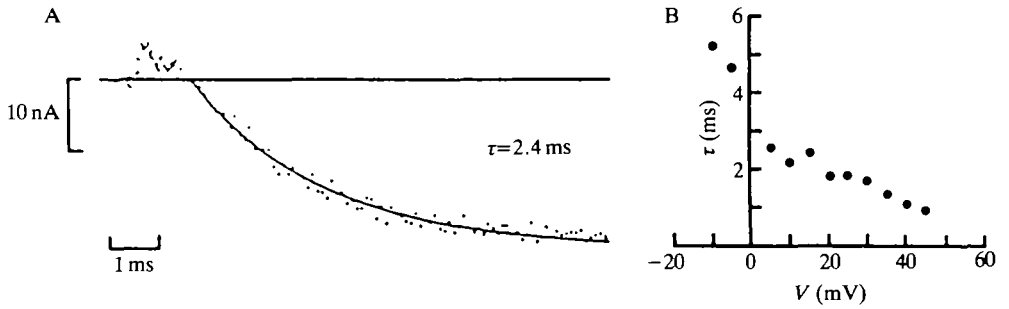

Fig. 6. Activation kinetics of Ba^{2+} current. (A) Ba^{2+} current recorded during a 50 ms pulse from −60 mV to +15 mV. A single exponential function fitted to the current cannot account for an initial delay before the channels open. The time constant of the late component was 2.4 ms. (B) Voltage dependence of the activation time constant (calculated as in A).

during the tail. The peak of the charge entry occurred at a pulse potential of +10 mV.

Activation and deactivation of Ba^{2+} currents

The early portion of Ba^{2+} current activation was obscured by a fast transient outward current. Nevertheless, the activation kinetics appeared sigmoidal. The entire time course could not be fitted by a single exponential since the rise developed only after an initial delay (Fig. 6A). The activation kinetics was voltage-dependent; the rise times were fastest at +25 mV with a τ value of 2.4 ms. Activation kinetics measured with Sr^{2+} or Ca^{2+} as the charge carrier was similar to that shown for Ba^{2+} in Fig. 6B.

The kinetics of channel closing or deactivation was estimated by measuring tail currents at the end of a depolarizing pulse. Deactivation, like activation, was voltage-dependent (Fig. 7). The decline of Ba^{2+} tail currents was best fitted with two exponentials (Fig. 7A). The later portion of the tail was fitted first. Subtracting this slow component (τ_{slow}) from the total tail current left the fast component (τ_{fast}). The sum of these two functions described the tail currents. For a closing voltage of −60 mV (the holding potential for most experiments), τ_{fast} was about 200 μs and τ_{slow} about 1.9 ms. In some cells, deactivation of Sr^{2+} currents was approximately 20 % faster than for Ba^{2+} or Ca^{2+} currents.

Inactivation of the divalent cation currents

The Ba^{2+} current through Ca^{2+} channels showed very little decline during 50 ms depolarizations. The current at the end of a 50 ms pulse was usually within 5–10 % of the peak level (Fig. 8). Similarly, changes in holding potential from −80 mV to −40 mV did not alter the current–voltage relationship for Ba^{2+} current or the activation kinetics. However, the decay of the current amplitude was greater with either Sr^{2+} or Ca^{2+} as the charge carrier. This was most readily observed during a 200–300 ms pulse. The amplitude decayed to as little as 10 % of the peak value for

a 300 ms pulse to +10 mV. The kinetics of this decay was well described by the sum of two exponentials. Unfortunately, a detailed analysis of the inactivation properties was precluded by partial obscuration of the inactivation by a contaminating outward current in many cases. This was made more prominent by the absence of Ba^{2+} and by the longer pulses required to observe the slow time course of the decay. Thus, it was difficult to determine reliably that portion of the current decay which represented true inactivation of calcium channels.

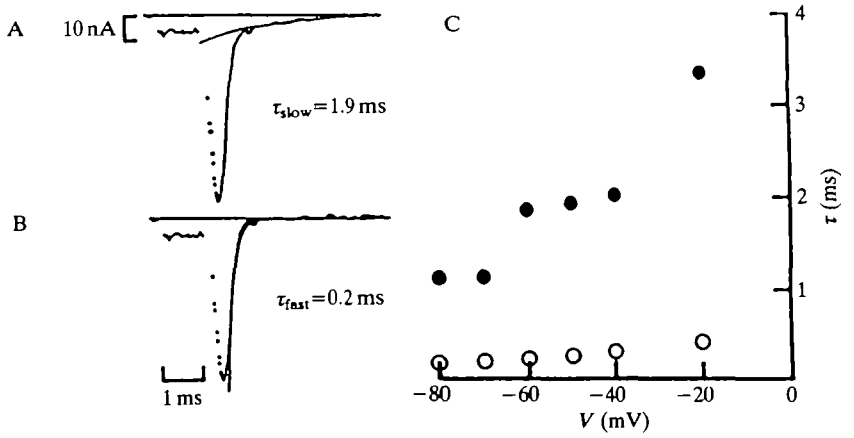

Fig. 7. Deactivation kinetics of the Ba^{2+} current. (A) Ba^{2+} current recorded during the last 1 ms of a 10 ms depolarization to +30 mV and tail current after the pulse. A single exponential function was fitted to the slow part of the tail, yielding a time constant of 1.9 ms and extrapolated back to the beginning of the pulse. (B) The slow exponential from A was subtracted from the tail current, leaving the 'fast tail'. Another single exponential was fitted to the fast tail, yielding a time constant of 0.2 ms. (C) Voltage dependence of the deactivation kinetics. The slow (●) and fast (○) time constants were plotted against the membrane potential.

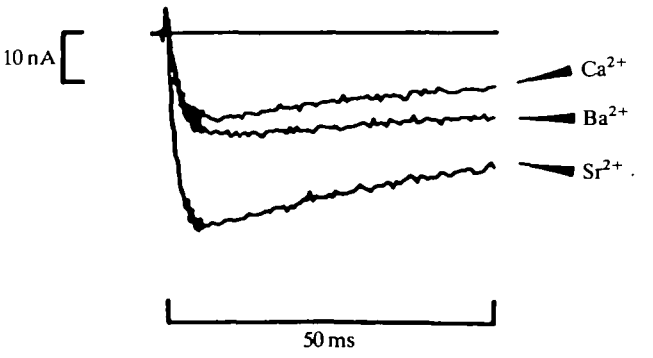

Fig. 8. Inactivation kinetics of Ca^{2+}, Ba^{2+} and Sr^{2+} currents. Currents were recorded from the same cell with 25 mmol l^{-1} Ca^{2+}, Ba^{2+} or Sr^{2+}, respectively, during 50 ms pulses to +15 mV from a holding potential of −50 mV.

Properties of the initial transient outward current

A fast, transient outward current obscured the early phases of Ba^{2+} current activation. Since specific blockers of sodium channels, such as saxitoxin or tetrodotoxin, do not affect Na^+ currents in *Hirudo medicinalis*, eliminating the external Na^+ only reduces *inward* I_{Na}. Residual intracellular Na^+ can still produce an *outward* I_{Na}. Under appropriate conditions (low external [Na^+]), depolarizing pulses to the sodium reversal potential still revealed a second component. This component was never inward for small depolarizing pulses and was an Off-current at the end of a depolarizing pulse. Ion substitution experiments failed to identify a reversal potential and the rising phase was too fast to be an outward I_{Na}. It seems likely that this represents a non-linear capacity current.

Discussion

One aim of the present experiments has been to describe the properties of calcium channels in leech neurones in culture; Retzius cells were of special interest because they form chemical synapses rapidly and reliably (Fuchs *et al.* 1982; Liu and Nicholls, 1989). In previous studies, calcium entry has been measured in these cells with conventional two-electrode voltage-clamp and with optical recordings using the Ca^{2+} indicator Arsenazo III (Stewart *et al.* 1989*a*; Ross *et al.* 1987). Although these techniques have provided information for correlating Ca^{2+} entry with transmitter release and growth, they are inadequate for measuring the kinetics of Ca^{2+} currents or for analysing how many types of calcium channel might be present.

The hybrid voltage-clamp method has offered certain advantages for approaching these problems. For example, the speed of the clamp and the small capacitative artefacts made it possible to measure more accurately the kinetics of activation and deactivation; also, isolation of the currents was improved since the technique permitted internal perfusion with Cs^+ and TEA^+ to block residual K^+ currents unaffected by external 4-AP and TEA^+. Nevertheless isolation of the Ca^{2+} current was never perfect, including the initial transient that masked early phases of Ca^{2+} current. One possibility is that this early transient represents gating currents for Ca^{2+} or Na^+ channels (Adams and Gage, 1976; Kostyuk *et al.* 1981). This interpretation is problematic due to the large current amplitude in some cells (Armstrong, 1981). Another possibility was that the transient was caused by outward monovalent cation movement through sodium channels (Hille, 1984).

The results obtained by the hybrid voltage-clamp method suggested the presence of a single type of calcium channel, rather than a multiplicity of channel types. The kinetics of activation and the two phases of deactivation can most simply be explained by one type of calcium channel with more than one closed state (Llinas *et al.* 1981; Tsien, 1983; Brown *et al.* 1983; Byerly *et al.* 1984; Llano and Bookman, 1986). In its properties the calcium channel of Retzius cells described here resembled others that activate at high voltages and inactivate slowly, such as the HVA, L or FD types of calcium channel seen in a variety of cells

(Carbone and Lux, 1984*a*; Nowycky *et al.* 1985; Armstrong and Matteson, 1985; Matteson and Armstrong, 1986). Retzius cell calcium channels were also similar to those of squid giant synapses and some other invertebrate neurones in their insensitivity to dihydropyridine agonists or antagonists (Augustine *et al.* 1987). With their slow and incomplete inactivation the calcium channels in Retzius cells behaved like those in nerve terminals or synaptosomes (Augustine *et al.* 1987; Lemos and Nowycky, 1989). This ensemble of properties, including high Sr^{2+} conductance and voltage-dependent activation and deactivation kinetics, set Retzius cell calcium channels apart from those found in other cells (Nachsen and Blaustein, 1982; Augustine and Eckert, 1984; Hille, 1984; Hess and Tsien, 1984; Hess *et al.* 1986; Tsien *et al.* 1987). In summary, Retzius cell calcium channels did not fall neatly into any of the conventional categories.

Unfortunately, patch recordings were too few to characterize Retzius calcium channels further at the single-channel level. The dearth of calcium channels in patch recordings was unexpected and cannot be simply explained. With peak macroscopic currents of approximately 20 000 pA and with cells having a membrane capacitance of approximately 1 nF (corresponding to 100 000 μm^2) one can estimate about one active channel per μm^2 (assuming that the single-channel current would be about 0.2 pA for such a peak depolarization). Single-channel pipettes covered approximately 1 μm^2 of the cell surface, from which one might expect most of the patches to have contained a channel. The small number actually observed could be explained by complicated infoldings of the cell membrane with calcium channels preferentially located in structures inaccessible to a patch pipette or by a non-homogeneous distribution of channels over the cell surface.

It was not possible by our techniques to assess whether different types of calcium channels exist in different regions of the Retzius cell, its soma, initial segment, axons and growth cones. A small population of such channels highly concentrated in one part of the cell would not necessarily give rise to distinctive currents measured under the present conditions. Optical recordings and loose patch-clamp experiments suggest that the voltage-sensitive calcium channels are not uniformly distributed but are more concentrated in the initial segment (Ross *et al.* 1987; Bookman *et al.* 1987; Nicholls and Garcia, 1989; Garcia *et al.* 1990).

In spite of such uncertainties regarding the numbers of calcium channel types and their distribution, these observations set the stage for further analyses of calcium channel function in relation to synapse formation and transmitter release by Retzius cells. The currents seen with whole-cell clamp can be compared with those observed by loose-patch clamp experiments and correlated with concentration changes measured by high-resolution optical imaging of calcium transients at newly formed synapses.

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