J. exp. Biol. 155, 435–453 (1991)
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SODIUM- AND CALCIUM-DEPENDENT EXCITABILITY OF EMBRYONIC LEECH GANGLION CELLS IN CULTURE

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Accepted 12 July 1990

Summary

Voltage-dependent Na^+ and Ca^{2+} inward currents underlying the action potential in cultured embryonic ganglion cells of the leech *Hirudo medicinalis* have been investigated using the gigaseal whole-cell current or voltage-clamp technique. Dissociated ganglion cells were isolated from 7- to 14-day-old embryos, and maintained in primary culture for up to 5 days. More than 95 % of the cultured cells had voltage-dependent K^+ currents and about 75 % of the cells had voltage-dependent inward currents. Action potentials of 60 mV amplitude and 4 ms duration, similar to those in embryonic nerve cells *in vivo*, could be recorded. Three types of inward currents occurred in these cells: (1) an initial Na^+ current, which activated and inactivated rapidly; (2) a second Na^+ current, which activated slowly and persisted during membrane depolarization, showing very little inactivation, and (3) a Ca^{2+}-dependent inward current. Both types of Na^+ currents were resistant to tetrodotoxin (TTX, 0.2–5 μmol l^{-1}). The Ca^{2+} current was also carried by Ba^{2+}, and was blocked by cobalt and cadmium. The fast Na^+ current was first expressed in cells from 8-day-old embryos, 1 day earlier than the Ca^{2+} current. Between days 8 and 14 the density of the fast Na^+ current increased from 22±3 to 51±6 μA cm^{-2} (±S.D., N=11), while the Ca^{2+} current grew from 10 μA cm^{-2} (N=2) to 15±4 μA cm^{-2} (N=10) during this time.

Introduction

During embryonic development, cells of the central nervous system (CNS) become electrically excitable and acquire the ability to generate action potentials. In early embryonic stages the ionic dependence of action potentials and membrane currents can differ from those of mature cells (Spitzer, 1979). The action potential

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Key words: Na^+-dependent excitability, Ca^{2+}-dependent excitability, leech ganglion cells, whole-cell current recordings, *Hirudo medicinalis*.

of *Xenopus* spinal cord neurones is initially long in duration and primarily dependent on an inward calcium current. During development, the contribution of sodium current to the action potential increases, while the calcium component decreases; the mature action potential is primarily sodium dependent (Spitzer and Lamborghini, 1976; Baccaglini and Spitzer, 1977).

Changes in the ionic mechanism of the action potential also occur in neuroblast cells of grasshopper embryos (Goodman and Spitzer, 1979). The inward sodium current and the first action potentials appear in the axon. Cell soma action potentials appear later, in which the underlying inward current is carried by both sodium and calcium ions. The patch-clamp technique (Hamill *et al.* 1981) has been used to investigate the appearance of voltage-dependent ion currents – mainly Ca^{2+}, Na^+ and K^+ currents – in neurones at different developmental stages (Bader *et al.* 1983; Ahmed *et al.* 1986; Barish, 1986; Ahmed, 1988*a*,*b*; Christensen *et al.* 1988; Gottmann *et al.* 1988).

Studies on the development of electrical excitability in neurones suggest that developmental changes in the inward currents are important for the maturation of the action potential. For the duration and shape of the action potential, however, K^+ outward currents appear to play a major role, as has been shown in embryonic *Xenopus* spinal neurones (Barish, 1986; Harris *et al.* 1988; O'Dowd *et al.* 1988). In adult leech ganglia, action potentials of several identified neurones are known to be Na^+-dependent (Nicholls and Baylor, 1968), and are unaffected by high concentrations of tetrodotoxin (Kleinhaus and Prichard, 1976; Johansen and Kleinhaus, 1986*a*). When the K^+ conductance is reduced by tetraethylammonium and 4-aminopyridine, slow Ca^{2+}-dependent action potentials can be elicited in some cells (Kleinhaus and Prichard, 1975; Johansen and Kleinhaus, 1986*b*). Recently, membrane currents underlying the action potentials of identified nerve cells in culture have been investigated using the two-electrode voltage-clamp method. The main currents recorded in cultured adult neurones were Na^+ and K^+ currents (Stewart *et al.* 1989*a*) and Ca^{2+} currents (Stewart *et al.* 1989*b*).

In the present study we have used the whole-cell configuration of the patch-clamp technique to investigate the appearance of action potentials and the timing of voltage-dependent inward currents in cultured embryonic ganglion cells of the leech. Some preliminary results have been communicated elsewhere (Deitmer and Schirrmacher, 1989; Schirrmacher and Deitmer, 1988).

Materials and methods

The experiments were performed on ganglion cells of 7- to 14-day-old leech embryos (*Hirudo medicinalis* L.). Maintenance and breeding of the leeches have been described elsewhere (Schirrmacher and Deitmer, 1989).

Cell culture

Segmental ganglia of the CNS were removed from 7- to 14-day-old embryos. At stages before completion of gangliogenesis, around day 11, only the anterior

ganglia were dissected. Ganglia were transferred to plastic Petri dishes (Falcon, 35 mm) that contained modified Leibovitz's L-15 medium with glutamine (see below) and were incubated for 10–40 min with collagenase/dispase (1 mg/2 ml, Sigma) added to the medium (Dietzel *et al.* 1986). After rinsing in medium without the enzymes, the ganglia were transferred to the culture dishes. The cells from the isolated ganglia were dissociated mechanically by gentle trituration in a fire-polished glass pipette. Cells were grown on uncoated glass coverslips (3 mm×3 mm) in L-15 medium with glutamine, supplemented with 0.2 ml of LPSR-1 (Low Protein Serum Replacement, Sigma), 0.6 g of glucose, 0.238 g of Hepes and 1 ml of gentamicin (Gibco) in 100 ml of L-15, according to Ready and Nicholls (1979). The cells were maintained at 20°C.

This isolation procedure provided about 20–50 unidentified ganglion cells on each coverslip. Isolated cells of 7- to 14-day-old embryos had diameters of 8–24 μm, determined optically with the help of a calibrated micrometer. Fig. 1 shows ganglion cells of 12-day-old leech embryos maintained in primary culture for up to 2 days. Cells were 15–20 μm in diameter. At this time cells had grown only small neurites.

Our attempts to isolate identified nerve cells directly from an embryonic ganglion with a suction pipette, a technique used to separate cells from the adult leech ganglion (Fuchs *et al.* 1981), were not successful. Furthermore, a method for identifying embryonic ganglion cells in primary culture, described by Gottmann *et al.* (1989), appears unsatisfactory. The authors assumed that Retzius neurones would occur among the 10 biggest cells in a culture dish containing cells isolated from three embryos. Since various sensory neurones have cell bodies of a similar size to Retzius cells (Schirrmacher and Deitmer, 1989), the probability of recording from sensory neurones rather than from Retzius cells seems high. We also selected ganglion cells with large cell bodies for our experiments and, therefore, these cells might well be either Retzius cells or sensory neurones.

Most recordings were made from spherical cell somata, to reduce space clamp problems that arise from an increase in cell surface caused by neurite outgrowth. Cell diameter was used to determine the surface area of the cell, which was presumed to be spherical. The calculated surface area was used to evaluate the ion current density.

Experimental procedure

Two to three hours after isolation, the cells adhered to the base of the dish and could be transferred to physiological solution containing (concentrations in mmol l^{-1}): NaCl, 112; KCl, 4; $CaCl_2$, 5; $MgCl_2$, 2; Hepes, 10; glucose, 11, adjusted to pH 7.4 with NaOH. To identify single ion currents, the normal saline was modified by ion substitution or by using specific channel blocking agents. To reduce K^+ conductance, tetraethylammonium (TEA^+) and 4-aminopyridine (4-AP) were added in exchange for an equimolar amount of Na^+. To isolate sodium currents, the extracellular solution contained (concentrations in mmol l^{-1}): NaCl, 100; KCl, 4; $CaCl_2$, 5; $MgCl_2$, 2; Cd, 0.5 (1); TEACl, 10; 4-AP, 2; Hepes, 10

(pH 7.4). Na^+-free solutions were prepared by replacing NaCl with *N*-methyl-D-glucamine (NMDG); pH was adjusted to 7.4 with HCl. Tetrodotoxin (TTX, 0.2–5 μmol l^{-1}) was added to the experimental solutions directly.

To unmask Ca^{2+} currents, $CaCl_2$ and $MgCl_2$ were replaced by $BaCl_2$ (concentrations in mmol l^{-1}): NMDG, 101; KCl, 4; $BaCl_2$, 7; TEACl, 10; 4-AP, 2; Hepes, 10 (pH 7.4). Ca^{2+} channels were blocked using $CdCl_2$ (1 mmol l^{-1}) or $CoCl_2$

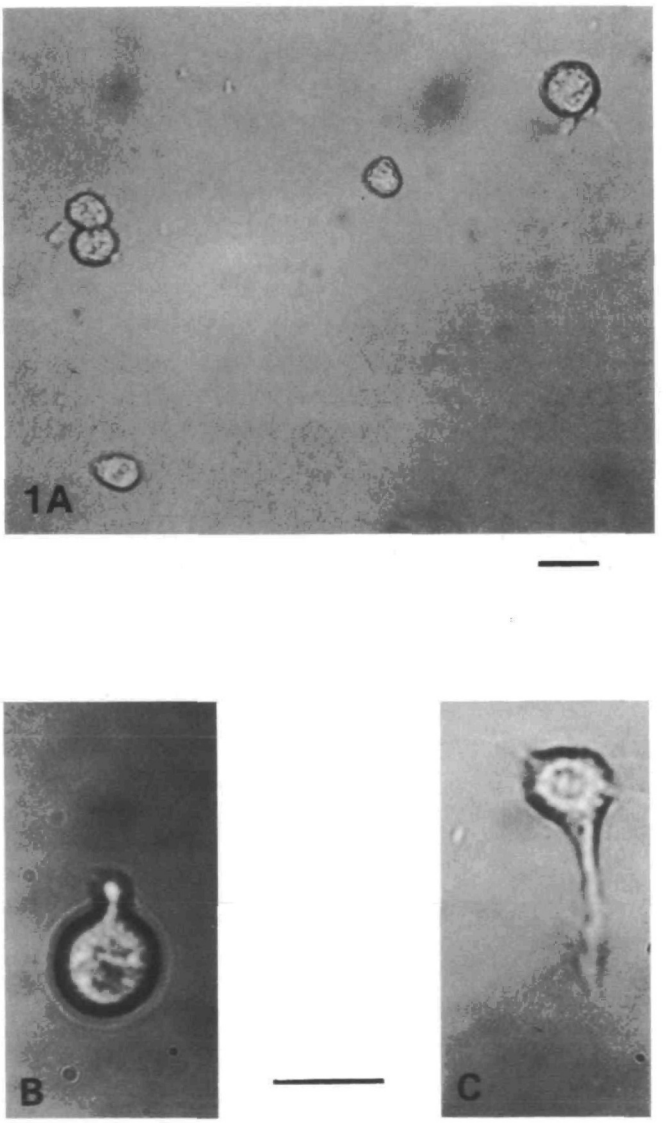

Fig. 1. Photomicrographs of ganglion cells isolated from a 12-day-old embryo maintained in primary culture for 6 h (A), 1 day (B) and 2 days (C). Note neurite and filopodial outgrowth in 1- and 2-day-old cells in culture. Scale bars, 12 μm.

($2\,mmol\,l^{-1}$) in exchange for $BaCl_2$ in order to maintain the divalent cation concentration.

Recording

Patch-electrodes were made from unfilamented glass tubes (Clark Electromedical Instruments, England, GC150–10) in a two-step pulling procedure (BB-CH, Mecanex SA puller, Geneva). A microforge (List-Medical L/M-CPZ101) was used to fire-polish the tip of each electrode. For whole-cell recording the glass pipettes were filled with a solution of the following composition (concentrations in $mmol\,l^{-1}$): KCl, 100 (or CsCl, 90; KCl, 10); TEACl, 10; EGTA, 0.1; Hepes, 10; Mg–ATP, 1; adjusted to pH 7.4 with KOH. The electrodes, when filled with internal solutions, had electrical resistances of 3–6 MΩ. The pipette was placed on the head-stage of an L/M EPC-7 (List electronics, FRG) patch-clamp probe and positioned with a hydraulic drive manipulator (Narishige, Japan). When the electrode made contact with the cell, gentle suction was applied to achieve a high-resistance seal of several gigaohms (Hamill *et al.* 1981), usually 5–50 GΩ. To gain access to the interior of the cell and to record whole-cell currents, the cell membrane was ruptured by further suction. The membrane input resistance of most cells was 2–5 GΩ. A pulse-generator (Digitimer D4030) delivered rectangular voltage pulses. Current and voltage signals were displayed on a digital two-channel storage oscilloscope, stored on floppy discs and recorded by a penrecorder (Gould).

Data analysis

All capacitive currents were adjusted using circuits within the EPC-7 amplifier. The membrane current signal was filtered with a frequency of 3 kHz (four-pole Bessel). Leakage currents linear with the command voltage and remaining capacity transients were subtracted by adding to the currents of interest ohmic currents recorded during voltage steps of equal amplitude but opposite polarity.

The voltage-clamp usually took much less than 1 ms to settle but, in some experiments, up to 1 ms (during the recording of slow currents) was accepted.

The experiments reported here and shown in representative figures, unless otherwise stated, were performed on at least five different cells.

Results

Embryonic ganglion cells in primary culture generated action potentials, which could be recorded in the current-clamp mode of the whole-cell configuration. Fig. 2 shows an example of such an action potential, elicited by depolarizing current pulses in a ganglion cell of a 12-day-old embryo, cultured for 1 day. The action potential had an amplitude of 60 mV and a duration of 4 ms in physiological saline. The average amplitude of the action potentials was 55 ± 4 mV ($\pm$S.D., $N=11$) at a mean resting potential of -42 mV. Addition of $1\,\mu mol\,l^{-1}$ TTX, an inhibitor of voltage-dependent Na^+ channels, to the bath solution did not change

the amplitude of the action potential. In Na^+-free solution, however, no action potential could be elicited. After replacement of the Na^+-containing solution action potentials reappeared. These action potentials were similar to those recorded from neurones of the embryonic ganglion *in situ* (Schirrmacher and Deitmer, 1989).

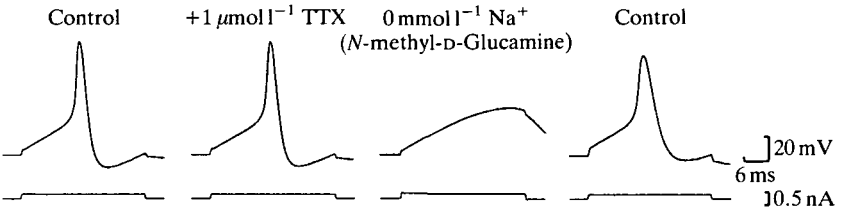

Fig. 2. Action potentials elicited in a ganglion cell (1 day in primary culture) of a 12-day-old embryo with constant current injection and recorded in physiological solution (Control), in a solution containing tetrodotoxin (TTX), in Na^+-free solution and in physiological solution (Control) again. The action potential is insensitive to TTX, but dependent on Na^+. The internal patch pipette solution contained (in mmol l^{-1}): K^+, 110; EGTA, 0.1; Hepes, 10; Mg–ATP, 1 (pH 7.4).

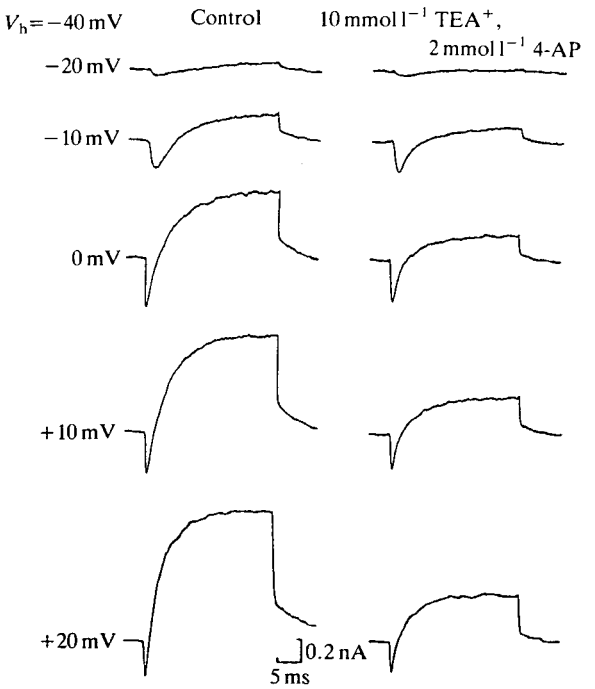

Fig. 3. Whole-cell currents recorded from a ganglion cell of a 9-day-old embryo in primary culture (1 day) in physiological solution (Control), and in a solution containing tetraethylammonium (TEA^+, 10 mmol l^{-1}) and 4-aminopyridine (4-AP, 2 mmol l^{-1}). Holding potential (V_h) −40 mV; in each case the potential during the voltage pulses is indicated.

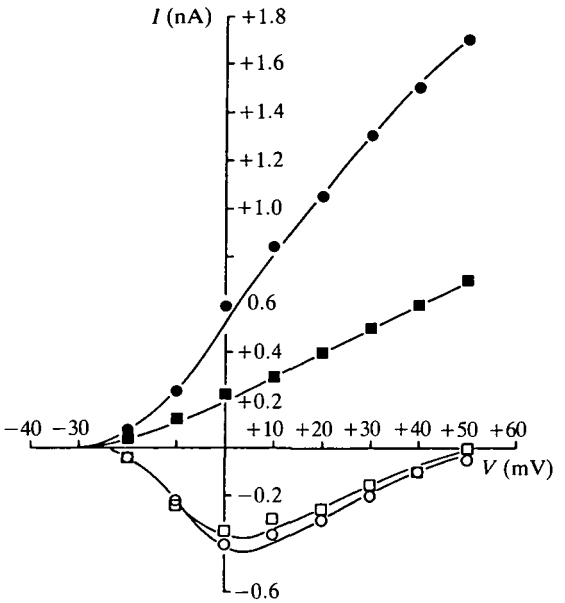

Fig. 4. Current–voltage (*I–V*) relationship of the initial inward current (○) and the following late outward current (●) in control solution. The K^+ outward current is reduced by TEA^+ and 4-AP (■), while the Na^+-dependent inward current (□) remains unchanged by these drugs. The internal patch pipette solution contained (in mmol l^{-1}): K^+, 110; EGTA, 0.1; Hepes, 10; Mg–ATP, 1 (pH 7.4).

Whole-cell currents

Membrane currents were elicited in normal saline from membrane holding potentials of −40 or −60 mV by step depolarizations of different amplitudes. Typically they were composed of both inward and outward currents (Fig. 3). Application of TEA^+ and 4-AP led to a reduction of the outward current, whereas the inward current was virtually unaffected. The remaining outward current, which was present only in some cells bathed in TEA^+ and 4-AP, could not be fully blocked even by the additional application of Ba^{2+}. The identity of this current is unknown.

The current–voltage (*I–V*) relationships are shown in Fig. 4. The maximum inward current reached 400 pA at a membrane potential of +5 mV in this cell.

Na^+ currents

To analyze the inward currents, TEA^+ (10 mmol l^{-1}) and 4-AP (2 mmol l^{-1}) were added to the extracellular solutions to reduce the K^+ conductance. In addition, the internal pipette solution contained TEA^+ (10 mmol l^{-1}) and CsCl (90 mmol l^{-1}) to block K^+ channels from the inside. To eliminate currents through Ca^{2+} channels the bath solution contained 1 mmol l^{-1} $CdCl_2$. When Na^+ was completely removed from the bath solution and replaced by *N*-methyl-D-glucamine, the inward current was reversibly abolished (Fig. 5). The

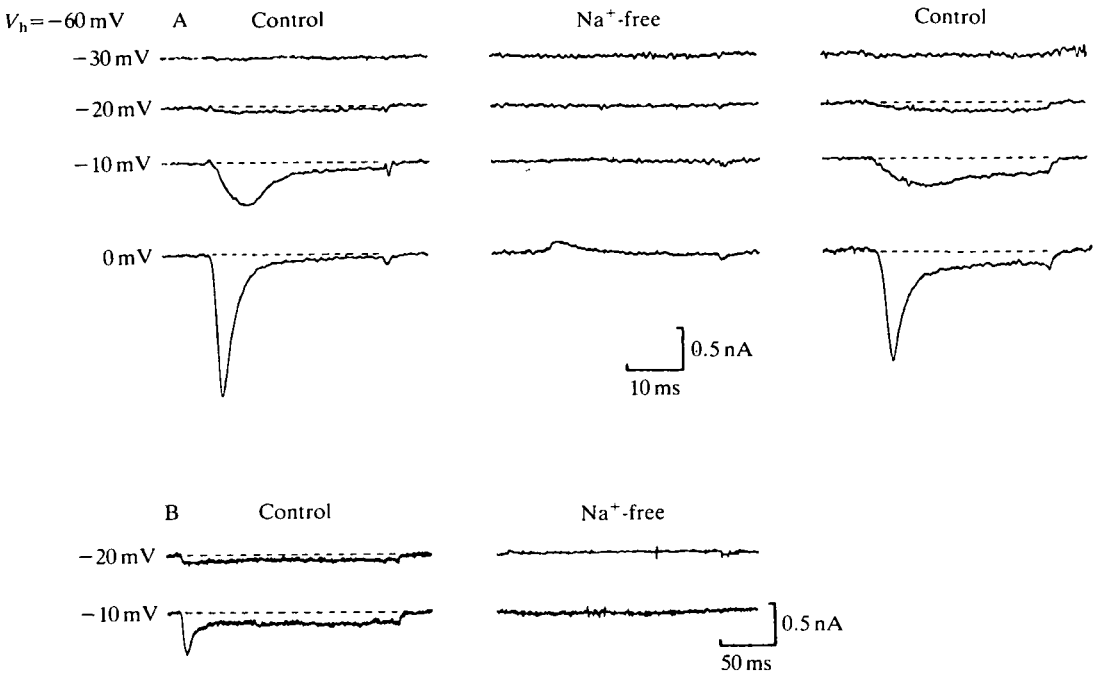

Fig. 5. (A,B). Recordings of the voltage-dependent Na^+ currents of a ganglion cell in primary culture (1 day) from a 12-day-old embryo on different time scales. In Na^+-free solution both the 'peak' current, which activates and inactivates rapidly, and the Na^+ component, which does not inactivate, are abolished. When the Na^+-free solution is replaced with control solution, these two Na^+ current components reappear. Ca^{2+} currents were blocked with 1 mmol l^{-1} Cd^{2+} added to the external solution. The holding potential was −60 mV, and in each case the potential during the voltage pulses is indicated. The internal patch pipette solution (in mmol l^{-1}): K^+, 100; TEA^+, 10; EGTA, 0.1; Hepes, 10; Mg–ATP, 1 (pH 7.4).

reversal potential near +60 mV, which is near the Na^+ equilibrium potential, confirmed that the inward current was carried by Na^+.

In some cells the inward current was composed of a 'peak' current, which activated and inactivated quickly (within a few milliseconds) and a 'steady-state' component, which inactivated very slowly. An example of recordings from a cell that showed inward currents made up of these two components is illustrated in Fig. 5.

Since the external solutions contained 1 mmol l^{-1} Cd^{2+}, currents through Ca^{2+} channels were fully blocked (see description of Ca^{2+} currents). The peak of this slow, Cd^{2+}-insensitive current was recorded at −5 to −10 mV.

Both current components disappeared in Na^+-free solution. When the control solution was replaced, both components reappeared (Fig. 5A). This indicates that both the fast and the steady-state inward current are dependent on Na^+. The slow component, however, did not inactivate even after several 100 ms (Fig. 5B).

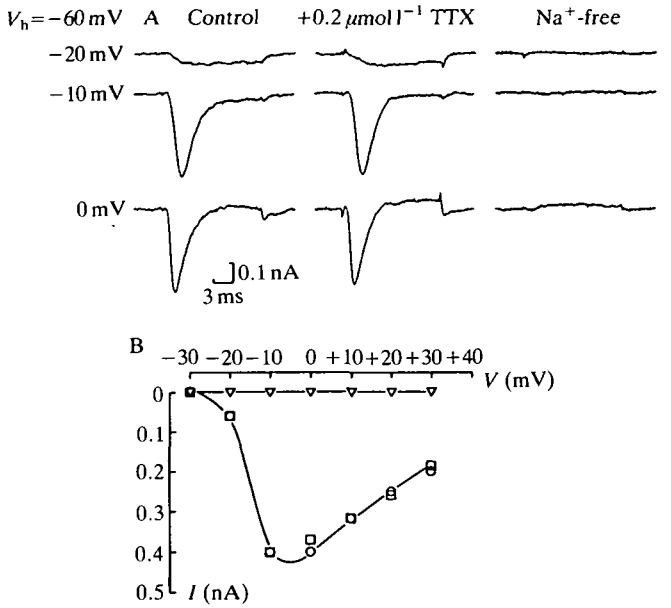

Fig. 6. (A) Voltage-dependent Na^+ currents recorded from a ganglion cell in primary culture (1 day) isolated from a 12-day-old embryo. Application of 0.2 μmol l^{-1} TTX blocks neither the fast Na^+ current nor the persisting Na^+ current. The holding potential was −60 mV, and in each case the potential during the voltage pulses is indicated. The internal patch pipette solution contained (in mmol l^{-1}): K^+, 100; TEA^+, 10; EGTA, 0.1; Hepes, 10; Mg–ATP, 1 (pH 7.4). (B) Current–voltage (*I–V*) relationship of the fast Na^+ current recorded in the same cell as A in control solution (○), in TTX-containing solution (□) and in Na^+-free solution (▽).

Effect of tetrodotoxin on Na^+ currents

Tetrodotoxin (TTX), a neurotoxin, selectively blocks Na^+ channels in many excitable tissues. In the leech, TTX does not affect the Na^+ action potentials in adult neurones (Kleinhaus and Prichard, 1976; Johansen and Kleinhaus, 1986*a*) or in embryonic neurones, either in culture (Fig. 2) or *in situ* (K. Schirrmacher and J. W. Deitmer, unpublished results). As is shown in Fig. 6, both peak and steady-state Na^+ currents of a voltage-clamped ganglion cell were unaffected by TTX. In Na^+-free solution these inward currents were completely abolished. The current–voltage (*I–V*) relationship shows the dependence of the fast inward current on the applied voltage (Fig. 6B). The maximum amplitude of the peak Na^+ current was 420 pA at −5 mV; the maximum steady-state Na^+ current amounted to 50–80 pA.

The application of TTX often resulted in a partial reduction of the fast Na^+ inward current. In five experiments a reduction of 9±5 % (±S.D.) of the amplitude by 1 μmol l^{-1} TTX was observed. In control experiments without TTX, a similar reduction was measured after 10–15 min (9±8 %, N=9), indicating some 'run down' of the Na^+ currents. Even in the presence of 5 μmol l^{-1} TTX (N=6), the decrease in current amplitude was the same as in the control experiments.

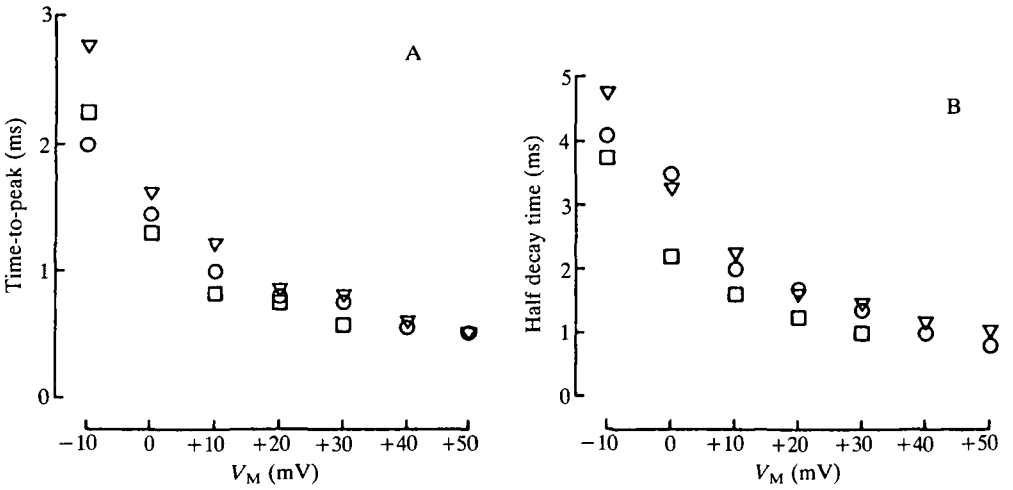

Fig. 7. Kinetic properties of the fast Na^+ current in cultured embryonic ganglion cells. (A) Activation (time-to-peak) and (B) deactivation (half-decay) of the current as a function of membrane potential (V_M) measured in control solution (○), TTX-containing solution (▽) and a solution in which Na^+ was replaced by Li^+ (□). TTX (1 μmol l^{-1}) and Li^+ affect neither activation nor deactivation of the Na^+ current. Data from three different cells produced very similar results (S.D. smaller than symbols).

Kinetics of the fast Na^+ current

The time course of the fast Na^+ current in embryonic ganglion cells was determined by analyzing the activation process (time-to-peak of the current) and the deactivation process (half-decay of the current). The Na^+ current activation was dependent on the step potential and was measured as the time between the onset of a voltage pulse and the peak current amplitude (Fig. 7A). At potentials more positive than +10 mV, activation was rapid: the time-to-peak was about 1 ms. In all cells tested TTX (1 μmol l^{-1}) did not affect the voltage-dependent activation of Na^+ channels. Replacement of Na^+ with Li^+ maintained the inward current with virtually the same activation rate. Neither the voltage dependence nor the activation process of the Na^+ current (N=132) changed in cells obtained from 7- to 14-day-old embryos.

The kinetics of Na^+ current decay was also voltage-dependent (Fig. 6B). The current was measured as the time between the onset of a voltage pulse and the time when the fast inward current had decayed to half-maximum amplitude. The deactivation kinetics increased with increasing depolarization between −10 mV and +50 mV. Deactivation was not affected by adding 1 μmol l^{-1} TTX or substituting Li^+ for Na^+.

Inactivation of Na^+ currents

The voltage dependence of Na^+ inactivation was investigated in double-pulse experiments. The voltage dependence of the steady-state sodium current inactivation was measured by applying conditioning prepulses in the range 10–60 mV

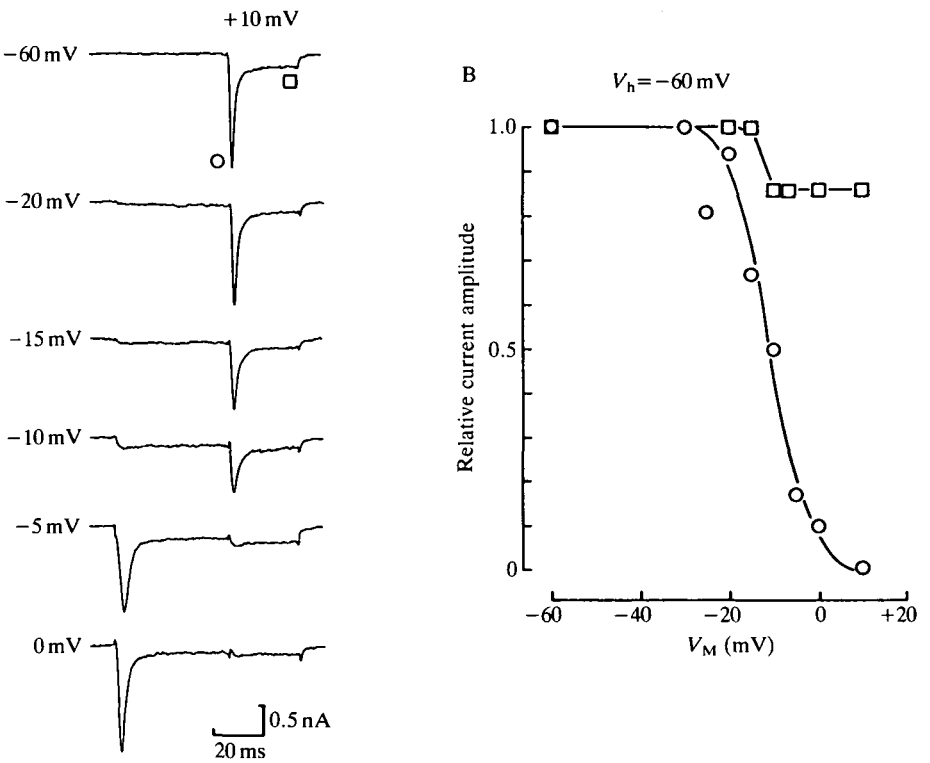

Fig. 8. Steady-state inactivation of Na^+ channels. (A) Na^+ currents from a cultured ganglion cell of a 12-day-old embryo. The holding potential was −60 mV, and in each case the potential during the prepulse (50 ms) is indicated and the potential of the test pulse was +10 mV. (B) Na^+ current amplitude during the test pulse compared to the peak amplitude without prepulse is illustrated as a function of the prepulse potential. Half-inactivation of the fast Na^+ current (○) occurs at $V_{0.5}$=−10 mV. The slow Na^+ current component (□) shows maximum inactivation of only 15 % at potentials more positive than −10 mV. The internal patch pipette solution contained (in mmol l^{-1}): Cs^+, 90; K^+, 10; TEA^+, 10; EGTA, 0.1; Hepes, 10; Mg-ATP, 1 (pH 7.4).

amplitude to the cell before stepping to a test potential of +10 mV (Fig. 8A). The peak and steady-state currents were then plotted as a function of the conditioning prepulse potential (Fig. 8B). Even at a prepulse potential of −20 mV, there was little inactivation of either Na^+ current component. The fast Na^+ current inactivated greatly with further depolarization and was reduced to about 10 % of its control value with a conditioning pulse to −5 mV. In this cell half the sodium channels were inactivated at a potential of −10 mV (on average at $V_{0.5}$=−13 mV, N=8). The voltage-dependence of the inactivation process was not significantly changed when the holding potential was changed to −80 mV ($V_{0.5}$=−15 mV, N=8).

The steady-state component of the Na^+ current, however, hardly inactivated

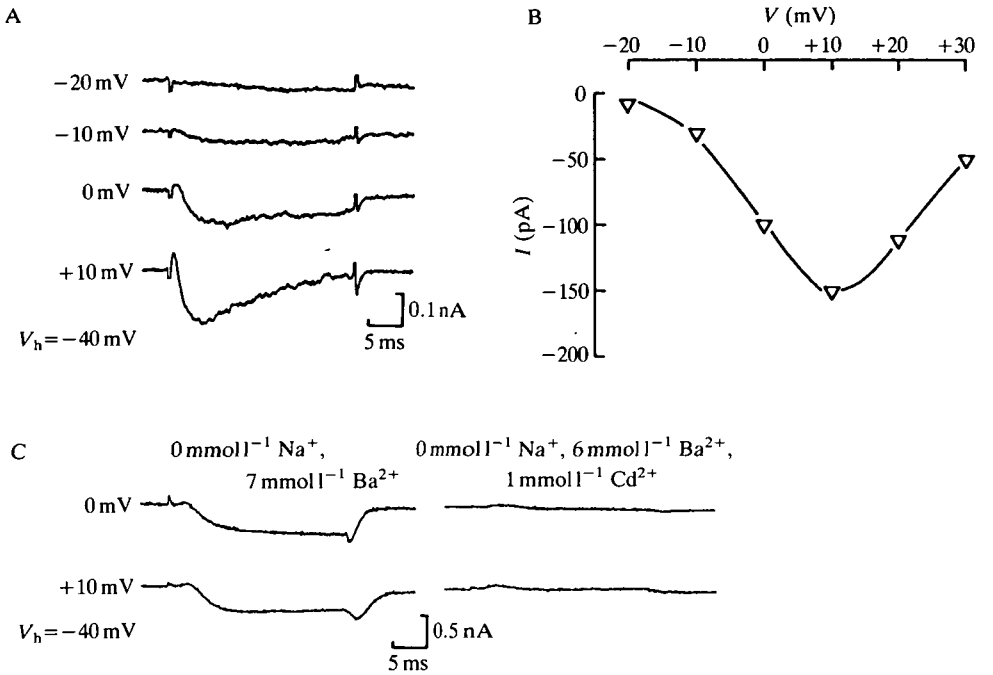

Fig. 9. Co^{2+}-sensitive, Ba^{2+}-dependent currents of a ganglion cell in primary culture (1 day) isolated from a 12-day-old embryo. (A) Currents in a Na^+-free solution containing 2 mmol l^{-1} Co^{2+} and 5 mmol l^{-1} Mg^{2+} (instead of Ca^{2+} and Mg^{2+}) were subtracted from currents in Na^+-free solution containing 7 mmol l^{-1} Ba^{2+} (substituting for Ca^{2+}). All solutions contained 10 mmol l^{-1} TEA^+ and 2 mmol l^{-1} 4-AP. The holding potential was −40 mV, and in each case the potential during the voltage pulses is indicated. (B) Current–voltage (*I–V*) relationship of the Ba^{2+} current in the same cell as A, showing a peak amplitude of 150 pA at +10 mV. (C) Voltage-dependent Ba^{2+} currents in another ganglion cell (1 day in primary culture) isolated from a 14-day-old embryo are blocked by 1 mmol l^{-1} Cd^{2+}. The holding potential was −40 mV. The internal patch pipette solution contained (in mmol l^{-1}): K^+, 110; Hepes, 10; EGTA, 0.1; Mg–ATP, 1 (pH 7.4).

(Fig. 8B). At prepulses between −5 mV and +10 mV the persisting Na^+ current was only reduced to 85 % of the maximum amplitude.

Ca^{2+} currents

Small Ca^{2+} currents are often masked by other ionic currents, mainly Na^+ and K^+ currents. To investigate these, therefore, the K^+ conductance was reduced with TEA^+, 4-AP and intracellular Cs^+, and Na^+ currents were eliminated by substituting *N*-methyl-D-glucamine for Na^+. Furthermore, Ca^{2+} and Mg^{2+} were replaced by Ba^{2+} to increase the current through Ca^{2+} channels. In addition, Ba^{2+} also blocks K^+ channels. Examples of a voltage-dependent Ba^{2+} current, indicating the presence of functional Ca^{2+} channels in the membrane, are illustrated in Fig. 9A. The inward currents, which remained in Na^+-free solution,

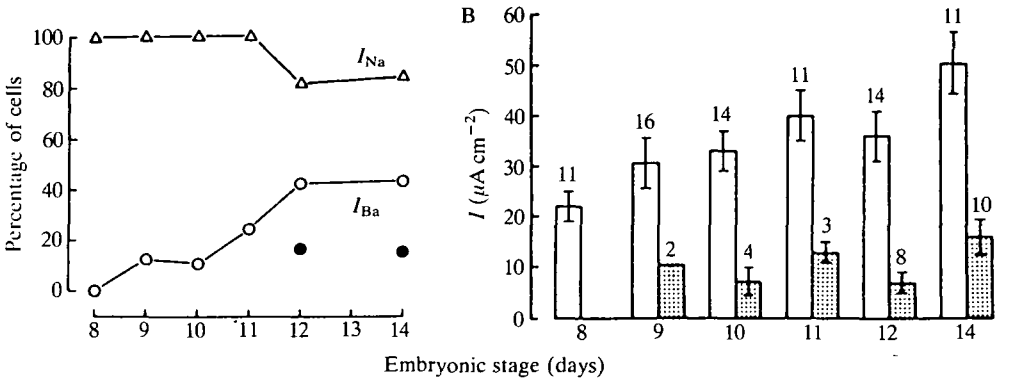

Fig. 10. (A) The percentage of cells expressing Na^+ and Ca^{2+} currents at different embryonic stages. All cells between days 8 and 11 have inward current carried by Na^+ (△), while the number of cells with Ca^{2+} currents (○) increases from day 9 to day 12. At day 12, 17% of the cells express only Ca^{2+} currents and no Na^+ currents (●). (B) Densities of Na^+ currents (open columns) and of Ca^{2+} currents (stippled columns) in cultured ganglion cells from different embryonic stages. Between day 8 and day 14, current densities increase, especially that of the Na^+ current. The change in current density of the Ca^{2+} current is smaller. The bars indicate mean values±S.D. (N=3–16).

activated and inactivated more slowly than the initial fast Na^+ currents. The recordings represent the Ba^{2+} current after subtraction of the currents in Ca^{2+}- and Ba^{2+}-free solution containing 2 mmol l^{-1} Co^{2+} and 5 mmol l^{-1} Mg^{2+} (Fig. 9A). Hence, even residual K^+ currents were subtracted. In this cell the peak amplitude of the Ba^{2+}-dependent inward current was 150 pA at +10 mV (Fig. 9B). Fig. 9C illustrates the complete blocking of these Ba^{2+} (Ca^{2+}) currents by cadmium.

Differences in the inactivation time course of the Ca^{2+} (Ba^{2+}) currents in the two cells illustrated in Fig. 9A,C are presumably due to the presence of overlapping K^+ currents, which were not fully inhibited.

Current densities during development

The earliest voltage-dependent inward current during embryogenesis, responsible for the development of electrical excitability, was expressed on day 8. Inward currents and action potentials were Na^+-dependent at these early stages. Ca^{2+} currents first appeared on day 9. Fig. 10 illustrates the appearance of Na^+ and Ca^{2+} inward currents in embryonic ganglion cells in culture. The inward current of all cells investigated was mainly carried by Na^+ (Fig. 10A). Between days 9 and 14 there was an increase in the number of cells with functional Ca^{2+} channels. The percentage of cells with Ca^{2+} channels increased from 12 to 42% during that time. From day 12 onwards, in 17% of the cells studied, only Ca^{2+} currents were found. All other cells had both Na^+ and Ca^{2+} channels or Na^+ channels only.

The densities of both Na^+ and Ca^{2+} currents were plotted as a function of developmental age (Fig. 10B). Between days 8 and 14 there was an increase in current density mainly in the Na^+ current. Na^+ current density grew from

22 μA cm^{-2} to 51 μA cm^{-2}, that is by a factor of 2.5, during that time (Fig. 10B). This presumably reflects mainly the large, fast Na^+ current and, to a small, unknown percentage, the slow, steady-state Na^+ current. The experiments indicate that more and more Na^+ channels are continuously incorporated into the cell membrane.

At day 9 the inward current dependent on Ca^{2+} (or Ba^{2+}) appeared, indicating the presence of the first voltage-dependent Ca^{2+} channels in the membrane. The density of the Ba^{2+} (Ca^{2+}) current increased by 50%, from 10 to 15 μA cm^{-2}, between day 9 and day 14 (Fig. 10B).

Discussion

In embryonic ganglion cells of the leech transferred into culture, three types of voltage-dependent ion channels were identified. Na^+ channels were expressed one day before the appearance of voltage-dependent Ca^{2+} channels. The initial Na^+ inward current showed fast activation and inactivation kinetics and was observed in most ganglion cells. In addition, another Na^+ current appeared; this current activated slowly and did not inactivate. In 10–40% of the cells an inward current, which was dependent on Ca^{2+} (Ba^{2+}) and was blocked by Co^{2+} and Cd^{2+}, remained in Na^+-free solution. Activation and inactivation were slow in comparison to the fast Na^+ current.

In addition to the appearance of voltage-dependent inward currents in developing leech ganglion cells, we also detected voltage-dependent K^+ outward currents, which appeared prior to the inward currents, as found in neurones of grasshopper embryos (Goodman and Spitzer, 1979) and in cultured avian neural crest cells (Bader *et al.* 1983). An analysis of the different K^+ currents that occur in leech embryonic neurones would be worthwhile, to determine the contribution of K^+ conductances in shaping the developmental changes of the action potential, but this has not been addressed in the present paper.

Properties of the Na^+ currents in embryonic ganglion cells

The properties of the fast voltage-dependent Na^+ current of embryonic leech ganglion cells in culture are similar to those of many other vertebrate and invertebrate cells. Na^+ inward currents in ganglion cells of 8- to 14-day-old embryos activate at a membrane potential of about −20 mV and reach peak amplitude at about +10 mV, and their direction is reversed between +50 mV and +60 mV. The current–voltage (I–V) relationship and the steady-state inactivation are similar to those of snail neurones (Adams and Gage, 1978) and chromaffin cells (Fenwick *et al.* 1982). Differences from other neurones exist in the voltage dependence of the inactivation. Whereas Na^+ channels in leech embryonic ganglion cells can be activated from holding membrane potentials of −40 mV, certain portions of those in embryonic *Xenopus* neurones, for instance, are already inactivated (O'Dowd *et al.* 1988).

In addition, a second Na^+-dependent component was observed, which activated

more slowly and did not inactivate. This small non-inactivating Na^+ current was not observed by Gottmann *et al.* (1989) in isolated leech embryonic ganglion cells. It is possible, however, that this Na^+ current occurs only in some types of cells, which Gottmann *et al.* (1989) did not investigate. This non-inactivating Na^+ current of embryonic ganglion cells might be analogous to the saxitoxin-sensitive, long-lasting, Na^+-dependent conductance found in *in situ* Retzius neurones of adult hirudinid leeches (Johansen and Kleinhaus, 1987). In other voltage-clamp studies on adult Retzius neurones, this type of current was, however, not observed (Stewart *et al.* 1989*b*).

Slowly inactivating Na^+ conductances have also been described in a variety of neurones: in stretch receptors of the lobster (Grampp and Sjölin, 1975), in giant neurones of *Aplysia* (Colmers *et al.* 1982), in squid axons (Matteson and Armstrong, 1982), in cockroach axons (Yawo *et al.* 1985), in hippocampal pyramidal cells (French and Gage, 1985), in frog muscle (Almers *et al.* 1983) and in a barnacle neurosecretory cell (Davis and Stuart, 1988). In embryonic chicken heart cells, an additional class of slowly inactivating Na^+ channels has been reported (Ebihara and Johnson, 1980; Ten Eick *et al.* 1984), which was absent in adult vertebrate heart cells (Brown *et al.* 1981; Follmer *et al.* 1987). It remains to be investigated if this slow Na^+ current appears predominantly in embryonic cells, and at what time in development it may disappear. The possible function of this slow Na^+ current during cell differentiation is unknown.

TTX-insensitivity of Na^+ currents

As in the cells of the adult leech (Kleinhaus and Prichard, 1976; Johansen and Kleinhaus, 1986*a*), Na^+-dependent inward currents of embryonic leech ganglion cells are insensitive to TTX (0.2–5 μmol l^{-1}). Na^+-dependent action potentials recorded from embryonic Retzius neurones *in situ* were also resistant to TTX (K. Schirrmacher and J. W. Deitmer, unpublished observations). Thus, TTX-insensitivity does not seem to be a characteristic of cultured cells; it is present in embryonic cells *in vivo* and remains unaltered during development. In embryonic rat skeletal muscle, in contrast, Weiss and Horn (1986) found a developmental change in TTX-sensitivity of two classes of sodium channels. In myoblasts, the embryonic precursors of myotubes, the voltage-activated Na^+ current was more resistant to block by TTX than that in myotubes. TTX-resistant sodium conductances are often a characteristic of immature, unexcitable cells (Spitzer, 1979). In some cultured nerve and muscle cells a distinct population of Na^+ channels is resistant to TTX (Fukuda and Kameyama, 1980; Bossu and Feltz, 1984). There seem to be differences in the structure of Na^+ channels, which manifest themselves in differential sensitivity to TTX.

Properties of the Ca^{2+} current

Many cells express different types of voltage-dependent Ca^{2+} channels with special functions (for a review see Deitmer, 1988). Single-channel recordings have revealed one type of Ca^{2+} channel in adult leech neurones (Bookman and Liu,

1987). Our results provide evidence for only one type of Ca^{2+} current for ganglion cells of 9- to 14-day-old leech embryos. When the holding potential was varied between −40 and −80 mV, the Ca^{2+} current still showed a one-peak current–voltage relationship, in contrast to the two-peak current–voltage relationship often associated with the presence of two types of Ca^{2+} currents (Deitmer, 1983, 1988).

Voltage-dependent Ca^{2+} (Ba^{2+}) inward currents, measured in Na^+-free solution, are blocked by Co^{2+} or Cd^{2+}; they are activated from a holding potential of −40 mV or more negative potentials, and inactivate slowly during a 30 ms depolarizing voltage pulse. The characteristics of Ca^{2+} currents in embryonic leech ganglion cells are similar to those of adult neurones (Johansen *et al.* 1987; Boev and Valkanov, 1988; Ross *et al.* 1988; Stewart *et al.* 1989*a*,*b*). In embryonic cells the peak amplitude is at potentials between +5 and +10 mV, and in the adult the peak is between +10 and +20 mV. Activation occurs at potentials more positive than −20 mV in embryonic cells and more positive than −10 mV in adult cells. Inactivation in both embryonic and adult neurones is slow and incomplete over tens of milliseconds.

Similar results have recently been reported by Gottmann *et al.* (1989). In sensory neurones and neural precursor cells of chicken embryos (Gottmann *et al.* 1988) and in some other vertebrate cells (Yaari *et al.* 1987; Beam and Knudson, 1988), however, two types of Ca^{2+} currents were present within a few hours in culture.

Na^+ and Ca^{2+} currents during early development

The earliest voltage-dependent inward current was measured in ganglion cells of 8-day-old embryos, and it was carried exclusively by Na^+. At this embryonic stage Retzius cells *in vivo* have grown neurites. The current density of the fast Na^+ current increases between day 8 and day 14 by a factor of 2.5, which is probably due to the addition of ion channels into the membrane. A sixfold increase of Na^+ current density was observed in neurones of mouse embryos between days 11 and 19 (Courad *et al.* 1986), and an increase as large as eightfold was reported in ventricle cells of 2- to 7-day-old chicken embryos (Fujii *et al.* 1988). This increase in current density was also thought to be due to an increased number of ion channels rather than to a change in the single-channel conductance.

At day 9 of leech embryogenesis, another voltage-dependent inward current, which is carried by Ca^{2+}, appears. Between days 9 and 14 the density of the Ca^{2+} current increases by 50 %, and the number of cells with functional Ca^{2+} channels increases. It may be speculated that Ca^{2+} is involved in developmental processes in the nervous system and in the regulation of various nerve cell functions (for reviews see Llinas, 1979; Hagiwara and Byerly, 1981). Changes in intracellular Ca^{2+} activity were shown to influence neuronal differentiation (Baccaglini and Spitzer, 1977) and the outgrowth of neurites (Anglister *et al.* 1982; Mattson and Kater, 1987).

The sequence of inward current appearance in the embryonic leech nervous

system resembles that of differentiating neurones from grasshopper embryos (Goodman and Spitzer, 1979), cultured cells from avian neural crest (Bader *et al.* 1983), dissociated rat diencephalic neurones (Ahmed *et al.* 1986) and motor neurones in the spinal cord of rat embryos (Ziskind-Conhaim, 1988). It differs, however, from that in various amphibian neurones, where the action potentials are first dependent on Ca^{2+} and later on Na^{+} (Spitzer and Lamborghini, 1976; Baccaglini and Spitzer, 1977), and from that in chicken sensory neurones (Gottmann *et al.* 1988), mouse embryonic neurones (Simonneau *et al.* 1985), cultured rat hippocampus neurones (Yaari *et al.* 1987) and muscle cells of *Drosophila* (Salkoff, 1985). One may conclude that timing and sequence in the appearance of voltage-dependent inward currents and outward currents are specific for each neuronal population.

We thank Mrs H. von Berg for her excellent assistance in maintaining the leech breeding colony. We are grateful to Professor Dr W. R. Schlue for his hospitality in his institute (Düsseldorf). This work was supported by the Deutsche Forschungsgemeinschaft by a Heisenberg-Fellowship (De 231/4–1, 4–2) and equipment grants (De 231/5–1,5–2) to JWD.

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