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VOLTAGE- AND STAGE-DEPENDENT UNCOUPLING OF ROHON-BEARD NEURONES DURING EMBRYONIC DEVELOPMENT OF *XENOPUS* TADPOLES

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SUMMARY

1. Electrical coupling of Rohon-Beard neurones in the spinal cord of *Xenopus laevis* has been studied from the time of closure of the neural tube (stage 20, 22 h after fertilization of the egg) to the free swimming tadpole (stage 49, 12 d old). Pairs of cells were examined by impaling both simultaneously with single micro-electrodes having current-passing and voltage-recording capabilities.

2. At the earliest time studied (stage 20), before Ca^{2+} action potentials are detected, cells are electrically coupled. Coupling coefficients as high as 0·6 can be recorded. Tests with small hyperpolarizing and depolarizing current pulses demonstrate that the coupling does not show rectification.

3. The coupling is voltage-dependent: depolarization or hyperpolarization of one cell with respect to another, above a threshold, causes relative uncoupling of the cells. The coupling coefficient falls to ~10% of its initial value when the difference in potential between the two cell bodies is ~75 mV. Cells usually become recoupled at the termination of the current pulse. Other, unidentified cells in the spinal cords of the same embryos show coupling that is not voltage-dependent.

4. Voltage-dependent uncoupling and recoupling persist when cells are depolarized by high K^+, and in the presence of 30 mM-Co^{2+}, suggesting that chemical synapses are not involved. They are also unaffected by addition of Rb^+, Cs^+ or TEA^+ to the extracellular solution, elevated levels of Ca^{2+}, or replacement of Na^+ with Tris or Cl^- with isethionate, suggesting that conductance changes in the surface membrane, such as anomalous rectification, are not responsible.

5. Lowering the intracellular pH with CO_2–HCO_3^- buffered saline does not abolish electrical coupling but appears to eliminate its voltage dependence.

6. Slightly later (e.g. stage 21), cells that do not yet produce Ca^{2+} action potentials while coupled will do so when their input resistance is increased by uncoupling them from their neighbours.

7. Later still (e.g. stage 23), cells make Ca^{2+} action potentials while coupled, and an action potential in one cell can trigger an action potential in other cells to which it is coupled. Ca^{2+} action potentials that do not bring other coupled cells to threshold for impulse initiation can transiently reduce the strength of coupling. Repetitive firing of these Ca^{2+} action potentials at a low frequency does not cause permanent uncoupling of the cells.

8. Rohon-Beard neurones become electrically uncoupled about stage 25 (early tail-bud, 28 h old). Coupling disappears around the time of appearance of the Na^+ component of the action potential, although coupling that is voltage-dependent or independent can still be seen between other, unidentified cells. No electrical coupling of Rohon-Beard cells was detected at later stages of development.

INTRODUCTION

Electrical coupling is widespread in many embryos (Sheridan, 1978). Cells with different developmental fates are initially connected by low resistance junctions, even at times when they have begun to express their distinctive phenotypes (Potter, Furshpan & Lennox, 1966; Sheridan, 1968; Spitzer, 1970; Blackshaw & Warner, 1976; Goodman & Spitzer, 1979, 1981). These cells subsequently become uncoupled, often at specific stages in their differentiation.

The Rohon-Beard cells of *Xenopus* tadpoles are a population of several hundred spinal neurones thought to be primary sensory cells (DuShane, 1938; Roberts & Hayes, 1977). Their early origin, large size (25 μm) and accessible dorsal location have facilitated electrophysiological investigations. Their birthdate and the development of some of their specific membrane properties have been described (Lamborghini, 1980; Spitzer, 1979*b*).

Here I report that Rohon-Beard neurones are electrically coupled to each other before the onset of other kinds of electrical excitability. The coupling shows a voltage dependence, such that polarization of one cell reversibly uncouples it from others. When these cells develop the capability of generating Ca^{2+} action potentials, an impulse in one cell can cause uncoupling from others. Rohon-Beard cells become permanently uncoupled about half a day later, around the time of appearance of the Na^+ current in the action potential.

Electrical coupling has been found to be sensitive to either internal H^+ (Turin & Warner, 1977, 1980; Bennett, Brown, Harris & Spray, 1978) or Ca^{2+} (Rose & Loewenstein, 1975) concentrations in many systems. Voltage-dependent coupling observed between *Xenopus* blastomeres is nearly abolished by lowered intracellular pH (Bennett, Harris & Spray, 1978; Spray, Harris & Bennett, 1979a). Similar pH reduction eliminates the voltage dependence of the coupling of Rohon-Beard neurones, but leaves them in a coupled state. A preliminary account of these findings has appeared (Spitzer, 1980).

METHODS

Embryos of *Xenopus laevis* were obtained by standard breeding procedures and staged by the criteria of Nieuwkoop & Faber (1956). Older preparations (> stage 25) were dissected as described previously (Spitzer, 1976). Younger preparations ($\leqslant$ stage 25) were made by pinning out the animal and removing the skin with tungsten needles and jewellers' forceps. The embryo was then viewed under a compound microscope equipped with a 40 × water immersion objective, and the dermatome layer above the spinal cord (Hamilton, 1969) was peeled off by teasing with micro-electrodes. Rohon-Beard neurones were visualized with Nomarski interference contrast optics, at 500 × magnification. At early stages the cells were identified on the basis of their size and position alone. From about stage 21 on, the cells were further identified by their ability to fire action potentials.

Cells were impaled with micro-electrodes filled with 3 M-potassium acetate (90–150 MΩ). Voltages

were recorded with WPI cathode follower amplifiers with current injection capability; neurones were depolarized or hyperpolarized by small current pulses passed through the recording electrode. Electrodes were selected for which adjustment of the amplifier prior to each impalement allowed passage of $\leqslant +0{\cdot}6$ nA or $\leqslant -0{\cdot}4$ nA without appreciable voltage change. This was accomplished by balancing out the potential drop across the electrode resistance; occasionally larger currents could be balanced. Single electrodes were often in balance for passage of both positive and negative currents of smaller amplitude. Electrodes had tip potentials of less than 3 mV. The output of the cathode follower current monitor, checked with a virtual ground, was accurate for these small currents both prior to and during impalements. There was often no detectable change in electrode resistance on impalement: the artifact resulting from the time constant of the electrode was nearly eliminated by adjusting the negative capacitance and 'bridge balance' outside the cell, and did not reappear upon entering the cell. The residual fast transients seen at the make and break of the current pulse were the same whether the electrode was in a cell or not. The difference in time constants of the electrodes and the cells would have permitted rebalancing of an electrode in a cell, in principle (Purves, 1981). However, data were rejected when the electrode resistance changed on impalement or during the subsequent course of the recording; once the electrode resistance started to change it often did so continually, making it difficult to adjust the balance rapidly enough to achieve accurate compensation. For some experiments a KCl-agar bridge was used between the bath and the chlorided silver wire leading to ground, to reduce shifts in junction potentials during the changing of salines. Voltages and currents were displayed on an oscilloscope and photographed, or registered on a Gould Brush 220 chart recorder. Interelectrode distances were measured with an ocular micrometer.

The preparations were perfused continuously with a saline of the following composition (mM): NaCl, 125; KCl, 3; $CaCl_2$, 10; HEPES buffer (pH 7·4), 5. Curare (D-tubocurarine, Calbiochem), 10^{-6} g/ml, was added to prevent muscle contraction. $CaCl_2$, $CoCl_2$, CsCl, RbCl, or TEACl were added to the saline without compensation for the increment in osmolarity. KCl was omitted when RbCl was used. When additional KCl was added to raise the final concentration to 10, 20 or 30 mM, the NaCl concentration was lowered by the same amount. Na^+ was replaced with Tris on an equimolar basis; lowered Cl^- concentrations were obtained by similar replacement of NaCl with Na isethionate. The extracellular pH was lowered to 6·5 by replacing the HEPES buffer with Tris or PIPES (adjusted with NaOH); the intracellular pH was lowered by replacing HEPES with 40 mM-$NaHCO_3$, and bubbling the solution with 100% CO_2, which is likely to bring the intracellular pH to ~6·5 (Turin & Warner, 1980). The concentration of $CaCl_2$ was reduced to 4 mM when using bicarbonate buffer, to prevent precipitation of $CaCO_3$. Experiments were performed at 19–22 °C.

RESULTS

Impalements of the cell bodies of pairs of Rohon-Beard neurones reveal that the cells are electrically coupled at the time of closure of the neural tube (stage 20). It is difficult to visualize these neurones at earlier stages of development. Depolarizing or hyperpolarizing current pulses injected into one cell cause a shift in potential not only of that cell but in another nearby as well; this shift is no longer recorded when the second electrode is withdrawn. The coupling coefficient ($\Delta V_2/\Delta V_1$) can be as high as 0·6 (mean $\pm$ S.D., $0{\cdot}25 \pm 0{\cdot}14$, $n = 28$). Although resting potentials as large as -80 mV are occasionally obtained, values of -30 to -60 mV are most often recorded, which probably reflect damage from the dissection or the impalement. Thus the coupling coefficient in the intact animal may be somewhat larger. The coupling is the same for small current pulses of both polarities, that is, no rectification is seen.

Voltage dependence of coupling

Many cells at the neural tube stage have not yet acquired the ability to generate action potentials, but they already display a different form of electrical excitability.

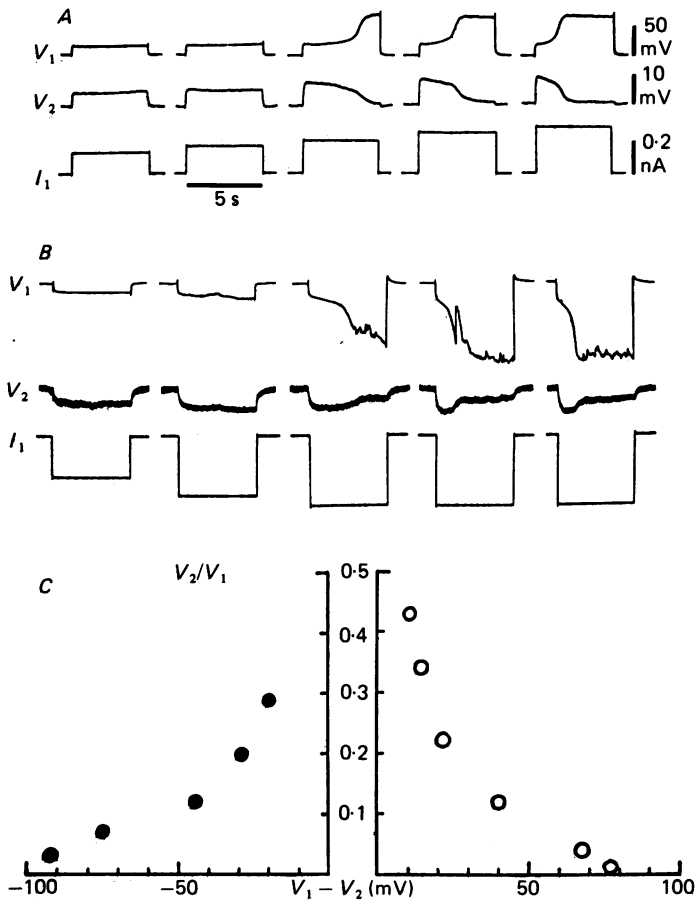

Fig. 1. Voltage dependence of electrical coupling of Rohon-Beard neurones. Records from two pairs of cells. *A*, injection of positive currents into one cell (I_1) depolarizes it (V_1) and another cell (V_2), 50 μm away. Larger current pulses produce an increase in the voltage difference between the cells, which is associated with relative uncoupling, after a latent period that decreases as the current strength is increased. Uncoupling leads to an increase in the input resistance of the first cell, indicated by the larger depolarization from the constant current pulse, while the depolarization of the second is decreasing. The cells become recoupled at the end of the current pulse. Resting potentials, −46 mV; stage 20. *B*, injection of negative currents into one cell demonstrates the same phenomenon in the hyperpolarizing direction. Same calibrations as for *A*. High gain and full capacitance compensation account for the thick V_2 trace in this and some subsequent Figures. Two cells 50 μm apart. Resting potentials (r.p.s) −52 mV, stage 20. *C*, the coupling coefficient at the end of each current pulse is plotted as a function of the difference between the potentials recorded from the cell bodies prior to uncoupling, for the cells illustrated in *A* (○) and *B* (●). The coupling coefficient decreases as the voltage difference increases.

Membrane polarization beyond a threshold level of about 30 mV causes a cell to become relatively uncoupled from others (Fig. 1*A*, *B*; $n = 20$). The injection of a constant current pulse in one cell leads, after some latency, to an increase in its input resistance, usually by a factor of 2–3. This is accompanied by a decrease in the voltage change of a second cell. The latency of the uncoupling decreases as the strength of the current pulse is increased. Standard current pulses of five seconds' duration were used in most experiments, but uncoupling can be obtained with half second pulses when they are sufficiently large. A residual 5–10 % of the initial coupling persists, even when the membrane potential is changed by large amounts. The uncoupling appears to be voltage-dependent, since the final coupling coefficient at the end of each current pulse is a function of the initial voltage difference ($\Delta V = V_1 - V_2$) between the two cells (Fig. 1*C*; nine of twenty coupled pairs were analysed in this manner). Upon termination of the injected current pulse the cells often become rapidly recoupled ($n = 11/20$; e.g. Fig. 1*A*), and the phenomenon can be repeated as long as the impalements can be maintained. The uncoupling does not depend on the absolute values of the membrane potentials, since it is seen at resting potentials ranging from -30 to -60 mV, and is present when the cells are depolarized by elevated levels of K^+ (see below). Furthermore coupling is observed only when the recorded resting potentials of both cells are similar. If the quality of one impalement is worse than the other, the cells are uncoupled unless the membrane potentials are brought to the same level by steady current injection. Hyperpolarizing, uncoupling currents consistently cause larger voltage changes than depolarizing, uncoupling currents of equal amplitude applied to the same cell, presumably due to non-linear properties of the cell surface membrane. Two-thirds of the pairs of impalements fail to demonstrate coupling between adjacent cells with the same resting potentials; this may be partly due to the damage caused by the dissection. Voltage-dependent uncoupling is not a global phenomenon in the spinal cord, since pairs of unidentified cells are coupled in a manner independent of similar values of ΔV.

Cells that have been uncoupled usually exhibit a noisy membrane potential, both upon hyperpolarization (e.g. Fig. 1*B*) and less prominently with depolarization (e.g. Fig. 4*B*). In the latter instance the relatively small noise is likely to be due to the effective increase in membrane resistance. The former involves a series of larger and more irregular fluctuations. The electrode resistance was the same before, during, and after impalements (see Methods). Accordingly, these fluctuations are unlikely to originate from the electrode, since they are not elicited by currents of the same magnitude when the electrode is withdrawn from the cell. Their activation by hyperpolarization suggests that they are not due to channels responsible for action potentials. They are unlikely to be synaptic potentials, since they persist in the presence of 30 mM-Co^{2+}. They may be the result of partial dielectric breakdown, when large hyperpolarizations are imposed on the negative resting potential.

Sometimes two cells appear to remain uncoupled briefly after the end of the current pulse ($n = 5/20$). With depolarizing pulses the injected cell shows some residual depolarization that decays to the resting potential, while the cell to which it was coupled is transiently hyperpolarized (Fig. 2). This residual depolarization may be due to leakage current around the current-passing electrode, whose contribution is detected only while the cells are uncoupled. At the same time, the leak current no longer diminishes the resting potential of the second cell, still uncoupled from the first, but coupled to a network of others. However, the voltage difference between

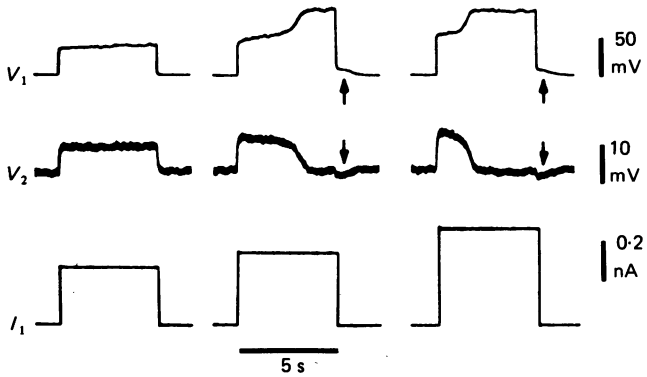

Fig. 2. Voltage-dependent uncoupling. Records from a single pair of cells. Cells are relatively uncoupled by injection of depolarizing current into one cell (V_1). At the end of the current pulse the injected cell remains depolarized briefly, while the other is transiently hyperpolarized (V_2; arrows). R.p.s, −50 mV; stage 20; cells 50 μm apart.

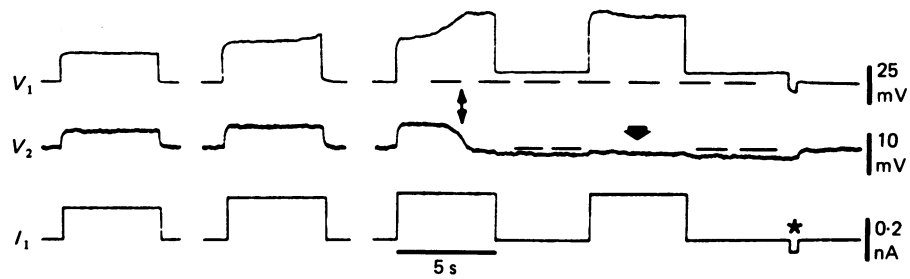

Fig. 3. Prolonged voltage-dependent uncoupling. Records from a single pair of cells, 50 μm apart. A depolarizing current pulse causes relative uncoupling (double headed arrow) that persists after the termination of the pulse. A second current pulse reveals that the cells are only weakly coupled (heavy arrow); the injected cell remains depolarized (V_1), while the other is hyperpolarized (V_2). A briefer hyperpolarizing current pulse brings the membrane potentials of the cells back to a common value (*), and the cells are recoupled (not shown). Dashed lines in this and subsequent Figures indicate the r.p. R.p.s, −54 mV; stage 20.

the two cells is insufficient to keep them uncoupled. In another pair of cells, injection of hyperpolarizing pulses causing uncoupling again reveals a residual depolarization of the injected cell after the end of the current pulse, while the hyperpolarization of the cell to which it was coupled decays more slowly (Fig. 1*B*). In both cases the membrane potentials relax to the rest level together.

Depolarizing or hyperpolarizing current pulses sometimes uncouple cells for a period long enough to test the hypothesis that the residual depolarizations and hyperpolarizations are associated with the uncoupled state (Fig. 3). Upon termination of the current pulse the first cell remains depolarized while the second is hyperpolarized ($n = 4/20$). Injection of a second current pulse reveals that the cells continue to be

relatively uncoupled. Later, hyperpolarization of the first cell brings the two membrane potentials close enough together for the cells to become recoupled and return to their common resting potential. Pairs of cells showing prolonged uncoupling (e.g. Fig. 3) can spontaneously convert to the rapidly recoupled variety, and vice versa, during the course of impalements lasting several minutes. Thus observations of rapid recoupling and prolonged uncoupling seem to be different manifestations of a single phenomenon. The cells exhibiting maintained uncoupling (e.g. Figs. 2, 3) have the same range of input resistances, in either the coupled or uncoupled state, as those showing rapid recoupling (e.g. Fig. 1*A*).

The results of the two-electrode experiments indicate that an increase in input resistance of the injected cell is associated with uncoupling from at least one other. Furthermore, they show that the prolonged depolarization, after termination of the current pulse, is associated with prolonged uncoupling. These observations permit the assay for voltage-dependent uncoupling in some experiments by single electrode measurements, since the appearance of the increase in input resistance can be used to indicate the presence of voltage-dependent uncoupling. In such experiments, brief constant current pulses superimposed on larger steady currents confirm that the depolarization associated with uncoupling involves a conductance decrease, and that this decrease is maintained while the cells remain uncoupled (Fig. 4*A*). This uncoupled state can be stable for as long as 5 min, the longest period tested, but the cells can be recoupled at any time by the injection of current pulses of sufficient magnitude (Fig. 4*B*). Extended depolarization of a cell (for 10, 20, or 40 s), which initially becomes rapidly recoupled after 5 s current pulses, often elicits prolonged depolarization and uncoupling after termination of the current pulse (six out of eight cases). Occasionally the cell into which current is injected seems to become serially uncoupled from more than one other cell or population of cells ($n = 4$). This is indicated by the appearance of additional inflections on the voltage trace during the injection of a constant current pulse, as the input resistance increases not once but twice (Figs. 4*C*, 5*A*).

Coupled cells have a threshold ΔV for uncoupling by depolarizing or hyperpolarizing pulses (Figs. 1–3), and a threshold for recoupling when the cells are in an uncoupled state (Figs. 4*B*, 5*A*). Positive current pulses of constant duration and increasing amplitude ultimately achieve a threshold depolarization at which the increase in input resistance occurs, which reflects uncoupling of one cell from its neighbours, and this can be summarized by an *I–V* relationship (Fig. 5*B*↑). When the cell remains uncoupled, negative current pulses of the same duration and increasing amplitude then lead to a threshold hyperpolarization at which the input resistance decreases, reflecting recoupling of the cell, and this result can be expressed as a second *I–V* curve (Fig. 5*B*↓). It is possible to travel around this loop repeatedly. The uncoupling and recoupling thresholds are not equal: the negative current pulses needed to recouple cells are smaller than the positive ones needed to uncouple them. This difference in current thresholds reflects the difference in voltage thresholds: for pairs of cells showing prolonged uncoupling, the ΔV_u causing uncoupling ($\Delta V_u = 30{\cdot}2 \pm 2{\cdot}0$ mV, mean ± S.D., $n = 4$) is often larger than the ΔV_m maintaining them in the uncoupled state ($\Delta V_m = 19{\cdot}5 \pm 6{\cdot}6$ mV; see Fig. 3). This point is supported by impalements of single cells, when the hyperpolarizing current injected

is abruptly decreased during the course of a long pulse. Currents that are too small to cause sufficient hyperpolarization to generate uncoupling are sufficient to maintain it once it is established. Similarly, a brief relaxation of the hyperpolarizing current leaves the cell sufficiently polarized to stay in the uncoupled state: the input resistance remains high and there is no latency in the voltage response when the current is stepped back (not illustrated).

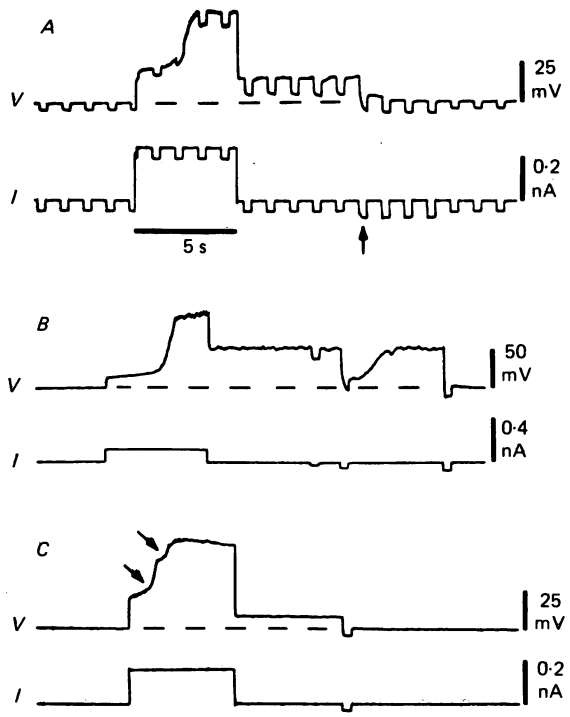

Fig. 4. Analysis of voltage-dependent uncoupling by single electrode impalements. Records from three cells. *A*, injection of a train of constant brief hyperpolarizing current pulses demonstrates the increased input resistance of the cell as it becomes uncoupled, and remains both depolarized and uncoupled. Increasing the strength of the hyperpolarizing current pulses brings the input resistance back to the original value (arrow), as the cell becomes recoupled to others. R.p. −60 mV; stage 21. *B*, the depolarized plateau associated with prolonged uncoupling is voltage sensitive: hyperpolarizing current pulses of increasing amplitude ultimately cause repolarization. R.p. −65 mV; stage 21. *C*, depolarization causes a two-step increase in input resistance (arrows), consistent with sequential uncoupling of the injected cell from two cells or populations of cells. R.p., −62 mV; stage 20.

Depolarization appears to produce a greater degree of uncoupling than hyperpolarization of the same cell by an equal or greater amount, in two-electrode experiments ($n = 9$). The coupling coefficient fell to 10% of its original value when one cell was depolarized to create a ΔV of about 60 mV (as measured in Fig. 1) for five pairs of cells, and when one cell was hyperpolarized to create a larger ΔV of about 90 mV for four pairs of cells. This may be an artifact of the impalements rather than

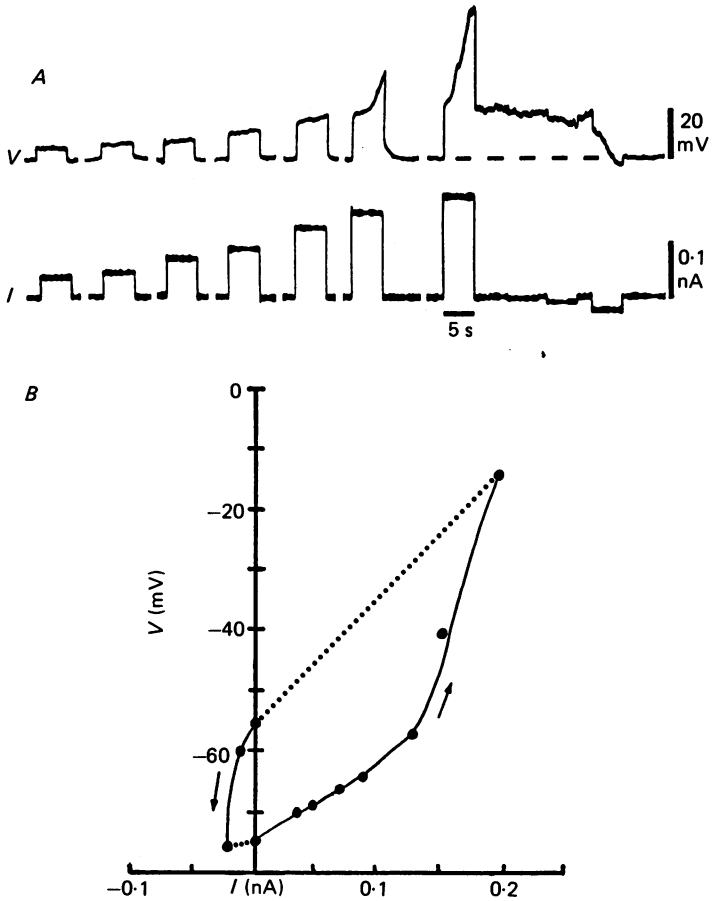

Fig. 5. Hysteresis of the voltage required for uncoupling and recoupling. *A*, records from a cell showing prolonged uncoupling. *B*, current–voltage relation for the same cell; the voltage at the end of the pulse is plotted as a function of the injected current. A current of 0·17 nA is needed to produce a depolarization of 24 mV that uncouples the cell from its neighbours. The cell remains uncoupled by a smaller depolarization (20 mV). A current of 0·02 nA is needed to produce the still smaller hyperpolarization of 8 mV that permits recoupling. Dotted lines indicate where the membrane potential goes at the end of current pulses that leave the cell in the uncoupled and recoupled states; curves were drawn by eye. R.p., −75 mV; stage 20.

a rectifying property of the uncoupling. When a cell is sufficiently polarized to become uncoupled from others, the membrane potential of the others often becomes more negative, now that the effect of any leak around the electrode of the injected cell is removed from the network of coupled cells (e.g. Figs. 2, 3). The magnitude of this effect will depend on the size of such leaks in the membrane created by the impalements. The result will be an apparent reduction in the residual coupling during depolarization, and an apparent increase in the residual coupling during hyperpolarization.

Voltage-dependent uncoupling was detected between cells separated by as much as 75 μm along the length of the spinal cord. The Rohon-Beard neurones are initially present in two rows on either side of the dorsal surface of the cord. Cells in one row are coupled in the voltage-dependent manner to cells in the other, as much as 120 μm across the cord ($n = 3$). Whether they are coupled directly or via other neuronal or glial cells is not known.

Ionic dependence of voltage-dependent uncoupling

Voltage-dependent uncoupling and recoupling persist when cells are depolarized by as much as 30 mV by the addition of KCl to the saline ($n = 7$), consistent with the observation of this phenomenon between pairs of cells with similarly low resting

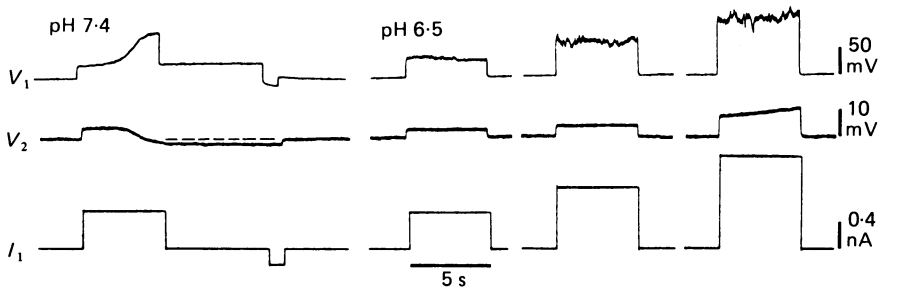

Fig. 6. The effect of low pH on voltage-dependent uncoupling of Rohon-Beard neurones. A pair of cells showing coupling and prolonged uncoupling at pH 7·4 remain coupled at pH 6·5, although the voltage dependence has been eliminated. The maximum voltage difference between the cells is four times that needed to cause uncoupling in normal saline. R.p.s, −44 mV; stage 20; cells 50 μm apart.

potentials in normal saline. The amount by which a cell must be depolarized to become relatively uncoupled from its neighbours does not appear to change, although uncoupling is initiated at less negative membrane potentials. As noted previously, the absence of an effect of high K^+ or the presence of 30 mM-Co^{2+} on voltage-dependent uncoupling supports the contention that chemical synapses are not involved. It is unlikely that voltage-dependent uncoupling is a property of the non-junctional membrane. Addition of 3, 10 or 40 mM-RbCl ($n = 4$) or 1 mM-CsCl ($n = 2$) to the saline has no consistent effect; these tests suggest that the decrease in conductance seen upon depolarization is not due to anomalous rectification (Hagiwara & Takahashi, 1974; Miyazaki, Takahashi, Tsuda & Yoshii, 1974). Replacement of Na^+ with Tris, Cl^- with isethionate, elevation of the Ca^{2+} concentration from 10 to 40 mM, or addition of 20 mM-TEA are all without effect, suggesting that four principal membrane conductances are not involved.

In contrast, perfusion with CO_2–HCO_3^- buffered saline (Turin & Warner, 1977) has a clear effect. This treatment, which probably lowers the intracellular pH to about 6·5, does not abolish electrical coupling ($n = 5$), but appears to eliminate its voltage dependence ($n = 11$; the presence of voltage-dependent uncoupling was evaluated in six cases with a single electrode impalement, as in Fig. 4). The shift to a lower

intracellular pH causes a consistent depolarization of the resting potential by an average of 25 mV ($n = 6$) which disappears upon returning to normal saline. The input resistance of the cell injected with current remains roughly constant ($n = 5$; e.g. Fig. 6), or decreases by a third to a half ($n = 5$). The coupling coefficient is the same as that of the cells in normal saline prior to uncoupling ($n = 3$), or decreased somewhat ($n = 2$; by 10 % and 40 %, viz. Fig. 6). At low pH, increasing the strength of the current pulse to three times that needed normally to initiate voltage-dependent

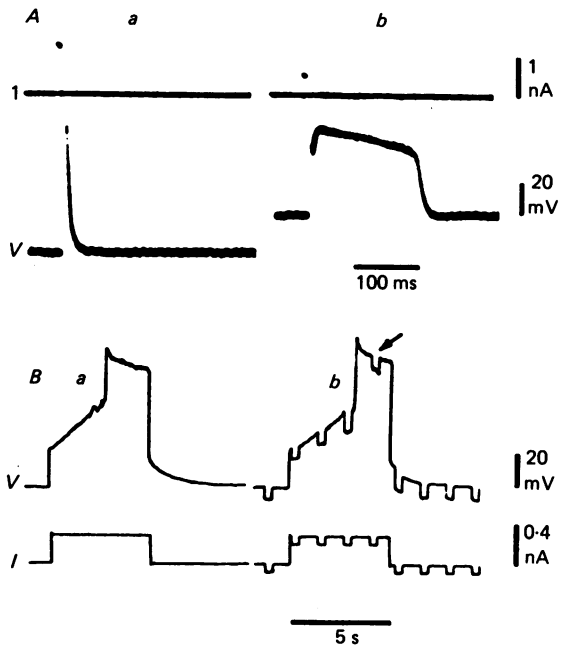

Fig. 7. Calcium action potentials are elicited following voltage-dependent uncoupling. Records from two cells. *A*, a cell unable to generate action potentials when depolarized from its resting potential by brief current pulses (*a*), does so after it is depolarized and uncoupled by steady current (*b*). R.p. −68 mV; stage 21. *B*, the increase in input resistance upon depolarization (*a*) is seen in the increased amplitude of the responses to small hyperpolarizing current pulses (*b*), but the Ca^{2+} action potential is associated with a decrease in input resistance (arrow). R.p. −68 mV; stage 21.

uncoupling does not elicit the phenomenon; the electrodes are not in balance for the passage of larger currents. Even when ΔV was four times that needed for uncoupling in normal saline, the cells failed to become uncoupled (Fig. 6); values up to one to two times were tested in four other cases and uncoupling was not observed. The effect of low pH is reversed upon return to normal saline. Lowering the pH of the saline to 6·5 with Tris or PIPES buffers, which presumably change only the extracellular pH, has no noticeable effect on voltage-dependent uncoupling ($n = 4$).

Calcium action potentials and voltage-dependent uncoupling

At about stage 21, Ca^{2+} action potentials can first be elicited. Superimposed on the voltage changes associated with uncoupling, there are voltage changes due to the

impulse. These involve an increase in conductance and are blocked by Co^{2+}. Occasionally no action potential is produced by a brief current pulse while the cell is at its resting potential (Fig. 7*Aa*). However, much smaller currents elicit an action potential when the cell has been uncoupled from its neighbours by a longer current pulse and its input resistance has been increased ($n = 3$, e.g. Fig. 7*Ab*). In some cases injection of a long current pulse not only uncouples the depolarized cell but also elicits

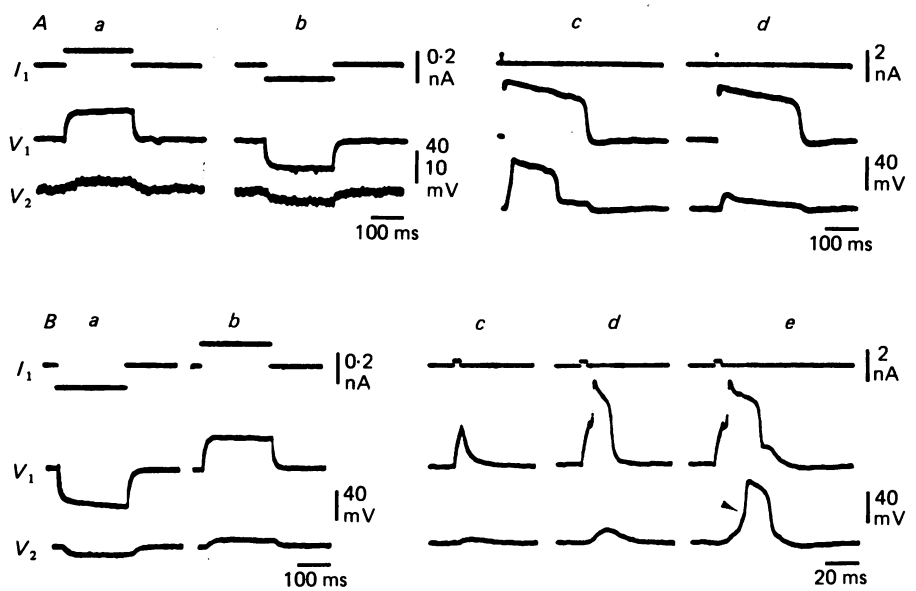

Fig. 8. Calcium action potentials are elicited while cells are coupled. Records from two pairs of cells. *A*, coupling of cells is revealed by current pulses that are subthreshold for initiation of voltage-dependent uncoupling (*a*, *b*); an action potential initiated in one cell initiates an action potential in the second (*c*). When the action potential in one cell fails to elicit an action potential in the second, the coupling coefficient is seen to decline during the course of the action potential (*d*). R.p.s − 50 mV; stage 23; cells 55 μm apart. *B*, coupling of cells is demonstrated by current pulses that are subthreshold for voltage-dependent uncoupling (*a*, *b*). Initiation of an action potential in one cell produces a coupling potential in a second (*d*), that can initiate an action potential in this cell also (*e*). Arrow indicates inflection where soma action potential arises from presumed neurite action potential. R.p.s − 60 mV; stage 27/28; cells 45 μm apart.

a brief train of events that are likely to be Ca^{2+} action potentials ($n = 5$; not shown). Square pulse analysis demonstrates that the input resistance of the cell is gradually increasing as the cell becomes uncoupled, and then abruptly decreases as the cell is rapidly further depolarized (arrow in Fig. 7*Bb*). Both the Ca^{2+} action potential and the associated resistance decrease are abolished by the addition of 10 mM-Co^{2+} to the saline ($n = 4$; not shown).

Within a short period of time Rohon-Beard neurones are capable of making Ca^{2+} action potentials when depolarized by brief current pulses at their resting potential, even though they are coupled to other neurones (Fig. 8*Aa*, *b*; $n = 18$), and the

coupling is voltage-dependent ($n = 4$; not illustrated). A Ca^{2+} action potential in one cell can cause depolarization of a second, bringing it to threshold, after a variable latency, for initiation of its own action potential ($n = 6$; e.g. Fig. 8*Ac*). The two action potentials are generally not of the same duration, and one of the cells exhibits a residual coupling potential after its action potential has ended. Repetitive firing of Ca^{2+} action potentials in the first cell at 0·5 Hz does not cause the cells to become uncoupled ($n = 6$). More often a Ca^{2+} action potential in one cell produces a depolarization of a second that is subthreshold for action potential initiation ($n = 12$; e.g. Fig. 8*Ad*). Although the initial depolarization of the action potential elicited by injected current spreads to other cells before the onset of detectable voltage-dependent uncoupling, the Ca^{2+} action potential can be of sufficient amplitude and duration that the coupling coefficient decreases during its course ($n = 5$; e.g. Fig. 8*Ad*). The cell generating the Ca^{2+} action potential can remain depolarized following termination of the impulse ($n = 6$; not illustrated), similar to the depolarization associated with prolonged uncoupling (Figs. 2, 3).

Cells remain coupled until about the time of acquisition of the Na^+ component of the action potential, and coupling is still voltage-dependent at these stages ($n = 5$). The ionic basis of these impulses was assessed on the basis of spike morphology, with long duration plateau responses taken to be Ca^{2+}-dependent, and shorter 'peak and plateau' responses taken as Na^+- and Ca^{2+}-dependent (Baccaglini & Spitzer, 1977). Only seven pairs of cells were observed to be coupled later than stage 25, when the Na^+ component of the action potential is usually first observed, and in six of these pairs both cells had long duration Ca^{2+} action potentials. The oldest example was observed at stage 27/28 (32 h of development), and is the only one in which one of the cells has what appears to be an initial Na^+ component in its action potential (Fig. 8*Bd*). The action potential in the first cell causes a small depolarization of the second, which is occasionally large enough to trigger a further depolarization, probably an action potential in a neurite (arrow in Fig. 8*Be*; Spitzer, 1976). This in turn elicits an overshooting action potential in the cell body.

Disappearance of coupling during development

Rohon-Beard neurones were usually not observed to be coupled after the time of appearance of the Na^+ component of the action potential, and coupling was not seen during subsequent development up to stage 49. By this stage many of the cells have died and disappeared from the dorsal surface of the spinal cord (Lamborghini, 1981). Depolarization of cells with large current pulses yields single action potentials, but the membrane potential in adjacent cells is unaffected (Fig. 9). Constant current pulses of long duration no longer elicit an increase in input resistance. The dissection may destroy some neurites. However, since many pairs of cells were examined at these later stages ($n = 42$), it seems unlikely that the site of coupling was consistently removed by the dissection, thereby creating the illusion of the loss of coupling. The disappearance of coupling is not a synchronous phenomenon for all cells in the spinal cord, since voltage-dependent uncoupling is present between other, unidentified cells after it is no longer detected between Rohon-Beard neurones. Still other cells are coupled in a voltage-independent manner at this time.

DISCUSSION

Voltage-dependent uncoupling

Rohon-Beard neurones are electrically coupled at early stages of development, in a voltage-sensitive manner. The coupling coefficient recorded between two cells is reduced when the membrane potential of one cell is shifted away from that of the other, in either direction, beyond a threshold of about 30 mV; the neurones become fully recoupled when their membrane potentials are made equal again. The observation that action potentials are not elicited by depolarization at these stages, despite the increased input resistance caused by uncoupling, provides further support for the initial absence of this membrane property (Baccaglini & Spitzer, 1977).

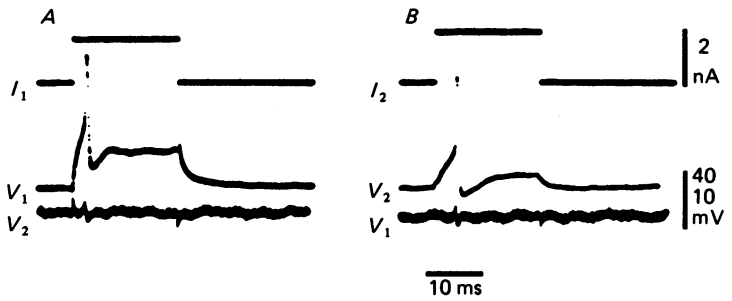

Fig. 9. Rohon-Beard neurones are uncoupled at late stages of development. Records from a single pair of cells. Action potentials elicited from either cell yield only a small field potential, recorded by the second electrode even when withdrawn from the cell. R.p.s − 68 mV; stage 49; cells 75 μm apart.

It is not known if Rohon-Beard neurone precursor cells are coupled throughout the period of proliferation from a small number of blastomeres (Jacobson, 1981). Since there is extensive coupling of cells in the neural plate (Warner, 1973), the coupling of these neurones could be residual. Alternatively, since the neurones migrate to their final position along the length of the spinal cord, they may become coupled after they have completed their migration. At these early stages Rohon-Beard cells are present in two rows, one on either side of the dorsal spinal cord. Cells within the same row were found to be coupled; cells in one row are also coupled to cells in the other. The size of the population of coupled cells is not known, but a single cell can be coupled to more than one other. It remains to find out if Rohon-Beard cells are coupled to other developing neurones or to glia. It seems likely that the coupling and its voltage-dependence are properties of gap junctions, although these have not been demonstrated morphologically.

The voltage-dependent uncoupling of Rohon-Beard neurones is similar in most respects to that of amphibian blastomeres (Spray, Harris & Bennett, 1979*b*, 1981*b*). However, the coupling of Rohon-Beard neurones is halved by a voltage difference of about 30 mV instead of 15 mV as in amphibian blastomeres. If the junctions are located along the neurites at some distance from the cell body, the actual trans-junctional voltage sufficient for uncoupling may be the same. A major difference is the

pH sensitivity of coupling: lowered pH has been reported to abolish coupling between blastomeres of *Xenopus* (Turin & Warner, 1977, 1980) and *Fundulus* embryos (Bennett *et al.* 1978; Spray, Harris & Bennett, 1981*a*). Lowered intracellular pH has the effect of greatly reducing or eliminating the voltage sensitivity of coupling between Rohon-Beard neurones, without altering the coupling itself. However, it has not been shown that the intracellular pH of these cells was reduced to 6·5 by the CO_2–HCO_3^- buffer, the level that abolishes coupling in other systems. Another possibility is that the coupling of these neurones is less pH sensitive than that of other cells, and abolished only at still lower values. In contrast, lowering the extracellular pH has no effect on coupling and its voltage sensitivity, although this treatment blocks Ca^{2+} action potentials when they appear within a few hours (Spitzer, 1979*a*).

It is not known whether the mechanism of uncoupling is voltage- or current-dependent. The relative uncoupling could be the result of the voltage difference between the cells, acting on a component of the junctional membranes or cytoplasm. Alternatively it could be the result of the current through the cell interiors and across the junctional membranes, leading to accumulation or depletion of specific ions. The same mechanism may not be involved in both the initiation and maintenance of the relatively uncoupled state, since the results of two-electrode as well as single-electrode experiments indicate that the voltage difference required to initiate neuronal uncoupling is greater than that needed to maintain it. One explanation could be that there is a considerable population of coupled cells, with the sites of coupling distant from the cell bodies. A large voltage change would then be required in a cell body to cause uncoupling, since much of the current would be shunted by the other cells to which it is coupled. A smaller voltage would suffice to maintain the uncoupled state, since more of the voltage shift imposed on the cell body would reach the sites of coupling. Another possibility is that there is hysteresis in the uncoupling mechanism, and a larger transjunctional voltage is needed to uncouple cells than to keep them uncoupled. Voltage-clamp studies of pairs of coupled blastomeres show that the transjunctional current prior to the onset of uncoupling is greater than that needed to keep cells in the relatively uncoupled state (Spray *et al.* 1979*b*). In this case only two coupled cells are involved, and the coupling site is isopotential with the site of current injection. A third possibility is that non-linear properties of the non-junctional membrane account for the hysteresis. A similar hysteresis exists in the I–V curve of the starfish oocyte membrane (Miyazaki, Ohmori & Sasaki, 1975).

The biological significance of voltage-dependent coupling is unknown. A brief change in the membrane potential of a cell is unlikely to activate the uncoupling mechanism. However, Ca^{2+} action potentials of long duration can cause a reduction of the coupling coefficient during their course, or lead to a prolonged residual depolarization, which may be due to voltage-dependent uncoupling. The voltage-dependent uncoupling of amphibian blastomeres is sufficient to prevent the movement of detectable amounts of Lucifer Yellow dye (MW 450) that occurs between two coupled cells (Spray *et al.* 1979*b*), consistent with a role of this phenomenon in restricting the intercellular movement of biologically significant molecules (Pitts, 1978). One possibility is that voltage-dependent uncoupling normally isolates neurones from each other so that they can express some form of independent development. Alternatively it could be a prelude to permanent uncoupling.

However the transient, partial uncoupling produced by Ca^{2+} action potentials could also be a consequence of Ca^{2+} influx. Elevation of intracellular free Ca^{2+} levels is reported to uncouple some cells (Rose & Rick, 1978). Repetitive firing of Ca^{2+} action potentials at a low frequency does not permanently uncouple these neurones, although calculations indicate that there is likely to be a substantial influx of Ca^{2+} (Baccaglini & Spitzer, 1977). The absence of uncoupling under these conditions may be due to rapid sequestering of Ca^{2+} (Lamborghini, Revenaugh & Spitzer, 1979), or the high concentration of Ca^{2+} required to uncouple.

Disappearance of coupling during development

Rohon-Beard neurones seem to become permanently uncoupled when the Na^+ component of the action potential appears (about stage 25), although there is some variability in the timing of these events. The cells have undergone considerable differentiation prior to electrical uncoupling, including their final mitosis, neurite outgrowth, and the onset of electrical excitability. This finding is similar to the observations made for DUM and MP3 ganglionic neurones of the developing grasshopper embryo (Goodman & Spitzer, 1979, 1981). These cells also develop their action potential mechanism while they are becoming uncoupled, but there is no obvious obligatory relationship between the two events. It is clear that amphibian spinal neurones can be completely dissociated and uncoupled from their neighbours at very early stages, and develop normally in culture in at least some respects (Spitzer & Lamborghini, 1976; Willard, 1980). Thus if coupling plays a role in development of these properties, it must do so prior to the neural plate stage at which the cultures are prepared. Uncoupling may be necessary for the differentiation of some other neuronal properties, however (e.g. neurotransmitter synthesis).

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