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DEVELOPMENTAL CHANGES IN THE INWARD CURRENT OF THE ACTION POTENTIAL OF ROHON-BEARD NEURONES

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SUMMARY

1. Rohon-Beard cells in the spinal cord of *Xenopus* tadpoles have been studied in animals from early neural tube to free-swimming larval stages. The onset and further development of electrical excitability of these neurones has been investigated in different ionic environments, to determine the ionic species carrying the inward current of the action potential.

2. The cells appear inexcitable at early stages (Nieuwkoop & Faber stages 18–20) and do not give action potentials to depolarizing current pulses.

3. The action potential is first recorded at stage 20. (A) The inward current is carried by Ca^{2+} at stages 20–25, since it is blocked by mM quantitites of La^{3+}, Co^{2+} or Mn^{2+} and is unaffected by removal of Na^+ or the addition of tetrodotoxin (TTX). (B) The action potential is an elevated plateau of long duration (mean 190 msec at stages 20–22). The duration decreases exponentially with repetitive stimulation. (C) The specific Ca^{2+} conductance (g_{Ca}) at the onset of the plateau of the action potential is $2{\cdot}6 \times 10^{-4}$ mho/cm^2. Calculations show that a single action potential raises $[Ca^{2+}]_i$ by more than 100-fold.

4. At later times (stages 25–40), the inward current of the action potential is carried by both Na^+ and Ca^{2+}: the action potential has two components, an initial spike which is blocked by removal of Na^+ or addition of TTX, followed by a plateau which is blocked by La^{3+}, Co^{2+} or Mn^{2+}.

5. Finally (stages 40–51), the inward current is primarily carried by Na^+, since the action potential is blocked only by removal of Na^+ or addition of TTX, and the overshoot agrees with the prediction of the Nernst equation for a Na-selective membrane. When the outward current channel is blocked and cells exposed to Na-free solutions, 67 % of cells at the latest stages studied were incapable of producing action potentials in which the inward current is carried by divalent cations.

6. The duration of the action potential decreases from a maximum of

about 1000 msec to about 1 msec during development. The maximum input resistance (R_{in}) decreases from ca. 1000 to 100 MΩ.

7. The calcium action potential may play a role in the development of excitability and the growth of the neurones.

INTRODUCTION

The action potential of most neurones depends primarily on an influx of Na^+ (Hodgkin & Katz, 1949), Ca^{2+} (Oomura, Ozaki & Maeno, 1961; Wald, 1972), or both of these ions (Koketsu, Cerf & Nishi, 1959; Geduldig & Junge, 1968). Although some egg cells are electrically excitable (Miyazaki *et al.* 1972, 1974*a*, 1975; Hagiwara, Ozawa & Sand, 1975; Shen & Steinhardt, 1976), amphibian eggs and cells from blastula and gastrula stages appear to be incapable of producing an action potential (Ito & Hori, 1966; Palmer & Slack, 1970; Warner, 1973). The aim of the experiments reported here was to investigate the acquisition of this neuronal property during development. The maturation of the action potential mechanism has already been studied extensively in tunicate, chick, and rat striated muscle cells (Takahashi, Miyazaki & Kidokoro, 1971; Miyazaki *et al.* 1972; Kano, 1975; Kano & Shimada, 1973; Sperelakis & Shigenobu, 1972; Kidokoro, 1973, 1975*a*, *b*), but such studies have not been reported for neurones.

Rohon-Beard cells, located in the spinal cord of amphibian tadpoles, are suitable for these experiments because of their large number and their accessibility (Spitzer, 1976). Moreover, these neurones are born very early (the last round of DNA synthesis occurs during gastrulation, 15 hr after fertilization of the egg), and are large enough to be impaled with an intracellular micro-electrode even at early stages of development (Spitzer & Spitzer, 1975; Spitzer & Baccaglini, 1975). Our results indicate that the ion species carrying most of the inward current during the action potential changes during development, from Ca^{2+}, to Na^+ and Ca^{2+}, and finally to Na^+. Calculations reveal that a single Ca^{2+} action potential could raise the intracellular concentration of Ca^{2+} more than 100-fold. This may be an interesting finding in view of the possible roles of calcium in events related to the growth and maturation of the cells.

Brief accounts of some of these results have already appeared (Spitzer & Baccaglini, 1975, 1976). The same developmental changes reported here also appear to occur in cell cultures of embryonic amphibian neurones (Spitzer & Lamborghini, 1976).

METHODS

Embryos of *Xenopus laevis* were obtained by conventional breeding procedures and preparations were dissected by methods previously described (Spitzer, 1976). Rohon-Beard neurones were visualized directly with Zeiss-Nomarski interference contrast optics. Embryos were staged according to the criteria of Nieuwkoop & Faber (1956); chronological age, in hours after fertilization of the egg, is also indicated in the text. Since the early stages of development are brief, recordings were made only during the first 2 hr following dissection.

Recording conditions. Cells were impaled with micropipettes filled with 3 M-potassium acetate (80–150 MΩ). An agar 3 M-KCl capillary bridge was used between the bath and the reference electrode. Voltages were measured by a cathode follower amplifier with current injection capability (WP Instruments-M4A) and displayed on an oscilloscope or pen recorder (Brush 220). Voltage ground has been indicated in all records. The neurones were depolarized or hyperpolarized by small current pulses passed through the recording electrode. The resting conductance and conductance changes were determined by measuring the voltage change produced by square pulses of hyperpolarizing current of 10^{-10} A or less. The duration of the action potential was measured as the time between the maximum rate of rise and maximum rate of fall. When determining the value of the overshoot of the action potential at late stages in solutions of different Na^+ concentrations, the preparation was perfused for 1 min in each solution prior to testing (flow rate 8 ml./min; chamber volume 0·5 ml.) (Spitzer, 1976). The cells were stimulated by brief current pulses (1·5 msec), and the value of the overshoot measured from records in which the action potential occurred after the termination of the current pulse. The following criteria were applied: (1) the cells had to have resting potentials more negative than −70 mV (Spitzer, 1976); (2) after the overshoot had been determined in the test solution, it had to return to within ± 4 mV of the original value in normal saline. In assaying the ability of cells to make action potentials in isotonic Ba, or Na-free plus TEA solutions, a criterion current pulse of at least 5 nA amplitude and 10 msec duration was used. This was more than 5 times the amplitude and duration of the stimulus that was necessary when responses were elicited successfully in normal saline.

Solutions. The preparations were perfused continuously. The composition of various salines is listed in Table 1. The presence of 5·0 mM Tris buffer (pH 7·4) made no difference; it was often omitted and the pH adjusted with small amounts of acid or base prior to an experiment. In Na-free solutions, tonicity was maintained by adjusting the molarity of the substituting ionic species (Tris HCl, choline Cl, $BaCl_2$, TEA-Tris HCl). Small amounts of blocking agents were added without compensation for increments in osmolarity. Curare (D-tubocurrarine, Calbiochem), 10^{-6} g/ml., was added to all solutions to prevent muscle contraction. Experiments were performed at 22° C.

RESULTS

Early developmental stages

Excitability. We are able to identify Rohon-Beard neurones by the morphological criteria of size and position as early as Nieuwkoop & Faber stage 18 (19 hr), when the neural folds are closely approximated and about to form the neural tube. The embryo develops from stage 18 to stage 20 in 3 hr. During this period, resting potentials more negative than −80 mV are frequently recorded, although values of −30 to −40 mV are more common.

TABLE 1. Composition (mmole/l.) of salines used in perfusion of preparations

	NaCl	NaAc	$CaCl_2$	$CaAc_2$	KCl	KAc	TrisHCl	Choline Cl	$BaCl_2$	TEA
(1) Normal	125·0	0	10·0	0	3·0	0	5·0	0	0	0
(2) 0 Na										
Choline Cl	0	0	10·0	0	3·0	0	5·0	125·0	0	0
Tris HCl	0	0	10·0	0	3·0	0	130·0	0	0	0
$BaCl_2$ (isotonic Ba)	0	0	0	0	3·0	0	5·0	0	110·0	0
TEA-Tris HCl	0	0	10·0	0	3·0	0	70·0	0	0	60·0
0 Cl	0	125·0	0	10·0	0	3·0	0	0	0	0
$\Delta[Na]_o$	40·0	0	10·0	0	3·0	0	85·0	0	0	0
	70·0	0	10·0	0	3·0	0	55·0	0	0	0
	90·0	0	10·0	0	3·0	0	35·0	0	0	0
	170·0	0	10·0	0	3·0	0	0	0	0	0

(3) Blocking agents (added to normal)

I_{Na}: TTX (10^{-6} g/ml.). I_{Ca}: $CoCl_2$ (10 mM), $LaCl_3$ (0·5 mM), $MnCl_2$ (2·0 mM). I_K: TEACl (10 or 20 mM).

Depolarizing current pulses of different durations fail to elicit a regenerative response (e.g. Fig. 1*A*). The failure to detect small inward currents could be due to the existence of outward K^+ currents (Fatt & Ginsborg, 1958; Werman & Grundfest, 1961; Hagiwara & Naka, 1964; Kidokoro, 1975*a*). However, the use of isotonic Ba solutions, in which Ba^{2+} may be expected to block potassium conductance (Sperelakis, Schneider & Harris, 1967) did

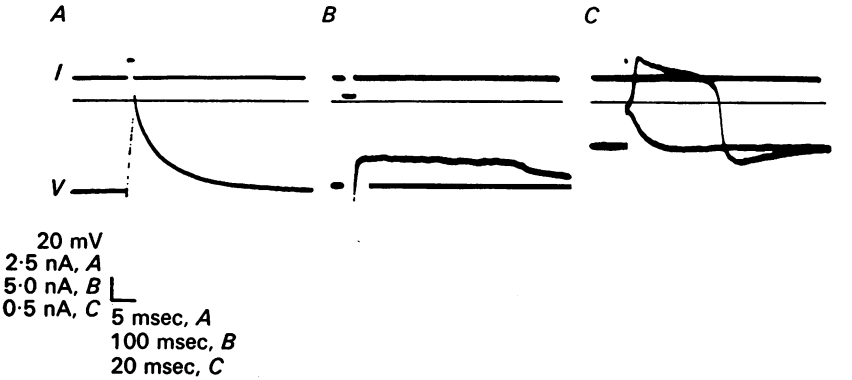

Fig. 1. Responses to electrical stimulation at early stages of development. Records from three cells. *A*, passive response to 2 nA current pulse; early stage 20. *B*, response to anode break stimulation; note long time scale; stage 23. *C*, action potential elicited by 0·4 nA current pulse. In two successive traces, the stimulus is sub- and suprathreshold. Stage 23.

not lead to the production of action potentials. The absence of electrical activity under these conditions suggests that the magnitude of any voltage-dependent inward current is small. Could conductances have been inactivated at these resting potentials? At a later stage they are not; however, there could be a developmental change in the voltage dependence of inactivation. If cells were coupled electrically at these stages, it might be difficult to elicit an action potential with point polarization. This possibility seems unlikely, since cells were loosely embedded in the neural tube, and were still inexcitable when they came up alone on the tip of the electrode. Occasionally, hyperpolarizing current pulses of long duration evoked what may be regenerative responses at the end of the pulse (Fig. 1*B*), following the absence of a response to depolarizing current pulses. These were up to 750 msec in duration. They fluctuated in length by several hundred msec and were eliminated reversibly by small amounts of La^{3+} or Co^{2+}. They may be early signs of excitability which are elicited by anode break excitation (Kidokoro, 1975*a*, *b*); another possibility is that they may be due to a dielectric breakdown of the membrane which is stabilized by the

presence of polyvalent cations. This latter possibility may be unlikely in view of the repeatability of the response.

Most Rohon-Beard neurones produce action potentials when examined at stage 20. An example of such an action potential is illustrated in Fig. 1C, showing the response to two successive stimuli; the first stimulus is sub-threshold. Above threshold, the regenerative response begins with a slow rate of rise that leads to a plateau; when the cell repolarizes it does so abruptly and is briefly hyperpolarized. Although both the rising and falling phases and the plateau were usually smooth, in three of forty cells sharp inflexions were observed (Fig. 2*A*3; arrows). This property may

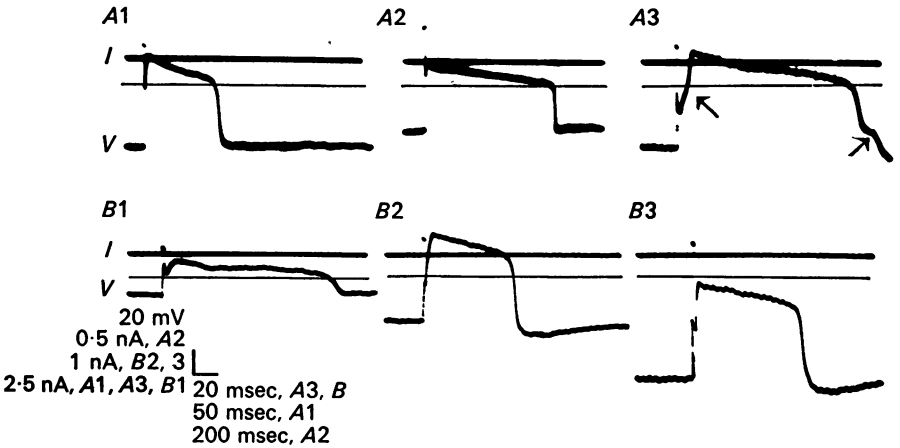

Fig. 2. Characteristics of action potentials at early stages. Records from six cells. *A*1, 2; variation in duration. *A*1: 150 msec, stage 21; *A*2: 1120 msec, stage 22. *A*3; variation in shape. Note points of inflexion (arrows), stage 23. *B*: responses at different resting potentials (R.P.). *B*1: 140 msec duration, RP −12 mV, stage 24. *B*2: 76 msec duration, R.P. −40 mV, stage 23; *B*3: 90 msec duration R.P. −88 mV, stage 23.

represent a transitional developmental state that is passed through rather quickly, but its low frequency of observation has prevented further analysis. Alternatively, it may be due to a particular geometry of the cells, since it is similar in appearance to the fractionation of the action potential observed in the pre-synaptic terminal of the squid giant synapse (Katz & Miledi, 1969). Some cells still appear inexcitable as late as stage 23.

The duration of these action potentials at stages 20–22 has been observed to range from 17 to 1100 msec, with a mean of 190 msec (forty cells). It appears to be relatively insensitive to changes in the membrane potential, since long action potentials have been recorded from cells with resting potentials that range from −12 to −88 mV (Fig. 2*B*). The variability in resting potential is likely to be a reflexion of the quality of the impalement.

Since the input resistance of the cells is greater at early stages than later (see below), a greater effect on the resting potential would be expected for a constant leak conductance introduced by the micro-electrode. Such observations suggest that the inward current channel does not have the rapid inactivation at low resting potentials, seen for Na channels in other preparations (Hodgkin & Huxley, 1952; Hille, 1967). The overshoot is variable, ranging from a few mV to 50 mV; it appears to have a maximum value in the range of -40 to -20 mV resting potential, and to decrease with resting potential values larger or smaller than this range.

Ionic dependence of the action potential. The ionic basis of the currents producing these regenerative responses was determined by changing the concentrations of extracellular ions, and by adding various blocking agents to normal saline. Several lines of evidence suggest that most of the inward

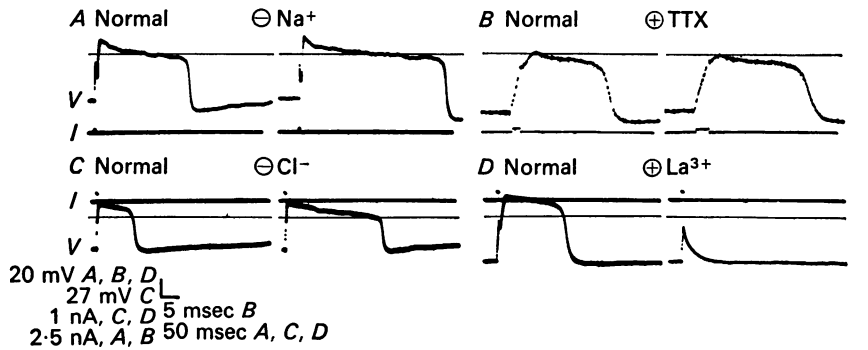

Fig. 3. Ionic dependence of action potentials at early stages. Records from four cells. Overshoot unaffected by (*A*) removal of Na^+ and replacement by choline, stage 25; (*B*) addition of TTX, stage 25; (*C*) replacement of Cl^- by acetate, stage 24. (*D*) Action potential eliminated by La^{3+}, stage 24. All changes were reversible. Note marked undershoots.

current is carried by Ca^{2+}. The first is the exclusion of a major role for other ions. Na^+ does not contribute substantially to the action potential at stages 20–25. Replacement of Na^+ with choline or Tris does not change the overshoot or diminish the duration of the action potential (Fig. 3*A*). However, an increase in duration is frequently observed, which may be due to a reduction in voltage-dependent potassium conductance, g_K, as a result of the removal of Na (Frankenhaeuser, 1962). Tetrodotoxin does not affect the action potentials at these stages (Fig. 3*B*); however, Na channels in other developing electrically excitable cells are also unaffected by TTX (Sperelakis & Shigenobu, 1972; Kidokoro, 1973). An outward Cl^- current does not contribute to the peak of the action potential, since

substitution of acetate for Cl^- does not change the overshoot of the response (Fig. 3*C*). The only effect of this procedure was an increase in the duration, suggesting a Cl^- current which has a significant effect at later times, possibly because the inward currents are smaller. We have not studied the process by which these ionic substitutions increase the duration of the action potential. A second line of evidence comes from the elimination of the action potential by small quantities of La^{3+}, Co^{2+}, or Mn^{2+} (Fig. 3*D*). These agents have been demonstrated to block voltage-dependent Ca^{2+} currents in other systems (Hagiwara & Naka, 1964; Hagiwara & Nakajima, 1966; Hagiwara & Takahashi, 1967; Hagiwara, Hayashi & Takahashi, 1969; Kidokoro, 1975*a*). Thirdly the overshoot varies linearly with the log $[Ca^{2+}]_o$, as demonstrated previously (Spitzer & Baccaglini, 1976). Calcium continues to be the principal ion carrying inward current until early stage 25. At stages 23–25 the duration of the action potential ranges from 5 to 940 msec (mean 90, forty-eight cells).

Calcium conductance. The membrane conductance at rest and during the plateau of the action potential was investigated by passing brief hyperpolarizing current pulses ($< 10^{-10}$ A) through the recording electrode. Input resistances were found to range from 200 to 1000 MΩ. Cell diameters are quite constant, and it is unlikely that this variability can be explained by variability in surface area. An example of these measurements is illustrated in Fig. 4. The conductance increases from *ca.* $2{\cdot}5 \times 10^{-9}$ mho at rest to $5{\cdot}0 \times 10^{-9}$ mho during the action potential. This finding eliminates the possibility that the action potential is due to a voltage-dependent decrease in g_K, characteristic of anomalous rectification. The resting conductance is actually somewhat smaller, since the current pulses are too brief in this instance to permit the potential displacement to reach steady state. As a result, the increase in relative conductance during the action potential is even larger. At these stages the cells are rather loosely adhesive in the embryonic spinal cord; once impaled, they can be removed from the tissue by raising the electrode. Cells isolated in this manner, without their processes, continue to give typical action potentials. Treating the cell of Fig. 4 as a sphere, and assuming a diameter of 20 μm (Spitzer & Baccaglini, 1976), the specific conductances are $2{\cdot}1 \times 10^{-4}$ mho/cm^2 and $4{\cdot}2 \times 10^{-4}$ mho/cm^2.

Could the change in conductance be due to an increase in voltage-dependent Ca^{2+} conductance, g_{Ca}? The value of g_{Ca} at rest is negligibly small (Spitzer, 1976). The addition of TEA prolongs the action potential in these cells, probably by blocking a voltage-dependent increase in g_K (Hille, 1967; Koppenhöfer, 1967). A Na-free solution containing TEA has no effect on the conductance at the onset of the plateau, however (Fig. 4*C*).

Since the resting conductance decreases only slightly, the full change in conductance is a reasonable measure of g_{Ca}. The mean g_{Ca} for eleven cells from stages 20–23 is $3{\cdot}1 \pm 1{\cdot}0 \times 10^{-9}$ mho ($R_{in} \geqslant 200$ MΩ). Mean specific conductance, calculated as in the previous paragraph, is $2{\cdot}6 \pm 0{\cdot}9 \times 10^{-4}$ mho/cm^2.

The calcium conductance declines during the course of the plateau of the action potential. This can be seen in Na-free solution, with TEA added to block g_K and prolong the response. In all six cells analysed in this way, the conductance declines exponentially (Fig. 5). The half-life for the decay of

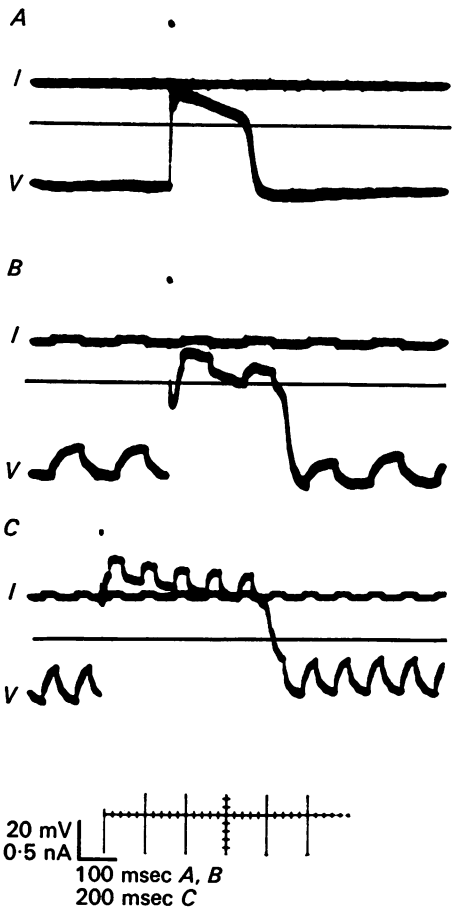

Fig. 4. Conductance measurements at onset of plateau of action potentials. *A*: normal action potential. *B*: superimposed response to brief hyperpolarizing current pulses in the same cell. *C*: as in (*B*), but in Na-free solution plus 20 mM-TEA; note different time scale. Conductance at onset of plateau is same in (*B*) and (*C*), $5{\cdot}0 \times 10^{-9}$ mho. Stage 21/22.

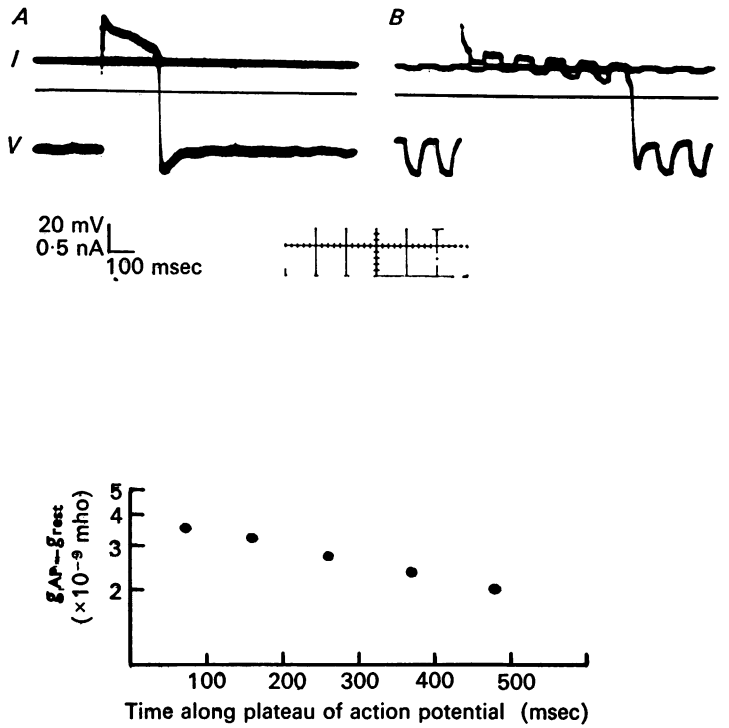

Fig. 5. Conductance change during the plateau phase of the action potential. *A*: normal action potential. *B*: superimposed response to hyperpolarizing current pulses in Na-free solution plus 20 mM-TEA, in the same cell. *C*: semi-logarithmic plot of data in (*B*). Stage 20.

g_{Ca} ranges from 30 to 600 msec, and is not a function of the initial magnitude of g_{Ca} for these cells (mean 3·1, range 2·0–3·8 × 10^{-9} mho).

What events are responsible for the termination of the action potential? The role of an activated conductance in repolarization of the membrane is indicated by three lines of evidence. First, the rate of repolarization of the action potential is often faster than the passive rate of decay (Figs. 4, 5). Secondly, the membrane is frequently hyperpolarized transiently when the cell is repolarized (Fig. 1*C*). Finally, TEA not only increases the duration of the action potential, but slows the rate of repolarization and reduces the hyperpolarization as well (Fig. 5). Thus, repolarization is due to both a decrease in g_{Ca} and an increase in g_K.

Duration changes with repetitive stimulation. A striking observation was that repetitive direct stimulation of the cells at 0·5 Hz, eliciting action potentials in normal saline, caused a decrease in duration of the action potential while the overshoot remained constant. In the absence of repeated

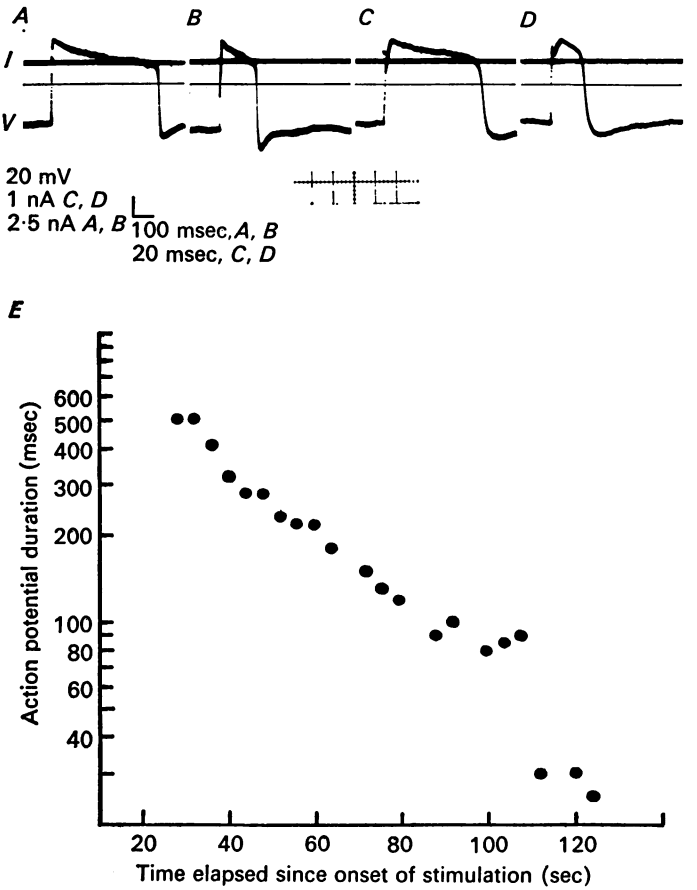

Fig. 6. Change in duration of the action potential in one cell, with repetitive stimulation at 0·5 Hz. Action potential after 30 sec stimulation (*A*), 1 min (*B*), 1·5 min (*C*) and 2 min (*D*). Note change in time base between *B* and *C*. *E*: semi-logarithmic plot of data for this cell. Note absence of decrease in overshoot. Stage 24.

stimuli the duration remained $\geqslant$ 80 % of its initial value when elicited by a single stimulus after periods of 2 min or more. An example of this phenomenon is shown in Fig. 6. The rate at which the duration decreased was exponential for each of five cells. The half-life for the decay of the duration ranges from 20 to 110 sec. These results are presented in Table 2. The ionic dependence of the action potential on influx of Ca^{2+}, assayed as described above, did not change during this period.

What causes the action potential to terminate after progressively shorter times? Both g_{rest} and the threshold remained constant, in at least four of the five cells. In principle, it might be due to a change in g_{Ca} or g_K.

TABLE 2. Relationship of g_{Ca} to duration of action potential (AP) and to changes in duration (to the nearest 10 msec)

Cell	Stage	g_{Ca} ($\times 10^{-9}$) (mho)	Half-life AP decay (sec)	Duration AP (msec)	
				Initial	Final
1	24	—	20	500	30
2	20	6·0	40	300	40
3	20	5·5	50	180	50
4	22	3·3	70	240	120
5	20	2·7	110	120	50

Measurements of g_{Ca} in Table 2 indicate that the value at the start of the plateau phase remains constant while the duration is shortening. It is not known if the rate of decay of g_{Ca} remains the same, since the normal decrease in the duration of the action potential has not been studied in the presence of TEA, which was used to measure g_{Ca}. When the half-life of the decay of the action potential duration is plotted as a function of either g_{Ca} or the log of the initially measured duration, linear relationships emerge from the small available data sample (Table 2). This indicates that the rate of shortening of the action potential may be linked to the amount of Ca^{2+} entering the cell. It is also possible that g_K is involved (see Discussion).

Intermediate developmental stages

A change in the appearance of the action potential is seen at stage 25 (28 hr). The long plateau response is replaced by an action potential which consists of a spike with rapid rate of rise, followed by a brief plateau (Fig. 7*A*1). This new shape was found in sixteen out of seventeen cells at stage 25, but the change had already occurred in five of forty-eight cells at stage 23/24. At stages 25–26 action potentials ranged from 2 to 46 msec in duration (mean 29 msec, twenty cells). The plateau becomes shorter with development, being reduced to a hump on the falling phase of the spike. However, the transition had apparently just occurred in two of the twenty four cells examined between stages 32 and 36, since the plateaus were still of long duration. The hump is barely detectable at stage 40, when the action potential duration ranges from 1·0 to 2·6 msec (mean 1·8, six cells).

The inward current responsible for the spike is carried by Na^+, since replacement of this ion with Tris or choline selectively eliminates it. In a small number of cases, it was still possible to obtain a regenerative plateau response with a larger current pulse under these conditions, in which the inward current was carried by Ca^{2+} (Fig 7*A*2). The spike is also blocked by

the addition of TTX, leaving a small, slow response due to the inward Ca^{2+} current (Fig. 7*B*2). This plateau can be blocked by La^{3+}, Co^{2+} or Mn^{2+} leaving the spike shape unchanged (Fig. 7*C*2). These observations indicate that, in normal saline, the inward current is carried by Na^{+} and Ca^{+2}.

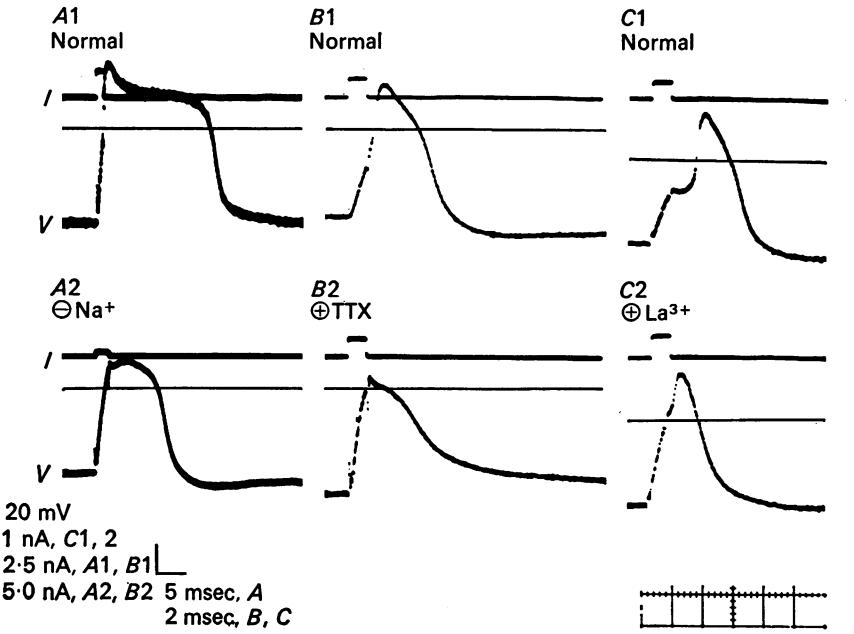

Fig. 7. Ionic dependence of action potentials at intermediate stages. Records from three cells. *A*1: normal action potential, consisting of initial spike and later plateau phase. *A*2: plateau component alone elicited by larger current pulse, in Na-free solution; Stage 25. *B*1: normal action potential with spike and plateau phases. *B*2: plateau component can still be elicited with larger current pulses in presence of TTX; stage 25. *C*1: normal action potential with spike and plateau phases. *C*2: plateau is selectively eliminated by La^{3+}; Stage 35/36.

Late developmental stages

The duration of the action potential at stages 42–45 ranges from 0·5 to 2·0 msec (mean 1·1, twenty-nine cells), and it did not change substantially during the next 2 weeks of development. By stage 42 (3·5 days) the inward current of the action potential is carried primarily by Na^{+}. Removal of Na^{+} or the addition of TTX abolishes the spike, while the presence of La^{3+}, Co^{2+} or Mn^{2+} does not affect its overshoot and duration (Fig. 8*A*). At stages 47–49 the overshoot was found to vary linearly with log $[Na^{+}]_o$ over a wide range of concentrations (40–170 mM). The least squares fit to the

observed values has a slope of 57·4 mV/ten-fold change in $[Na^+]_o$ (Fig 8*B*).

Has the cell membrane completely lost the ability to produce action potentials in which the inward current is carried by divalent cations at these late stages? This point was examined by passing depolarizing current into

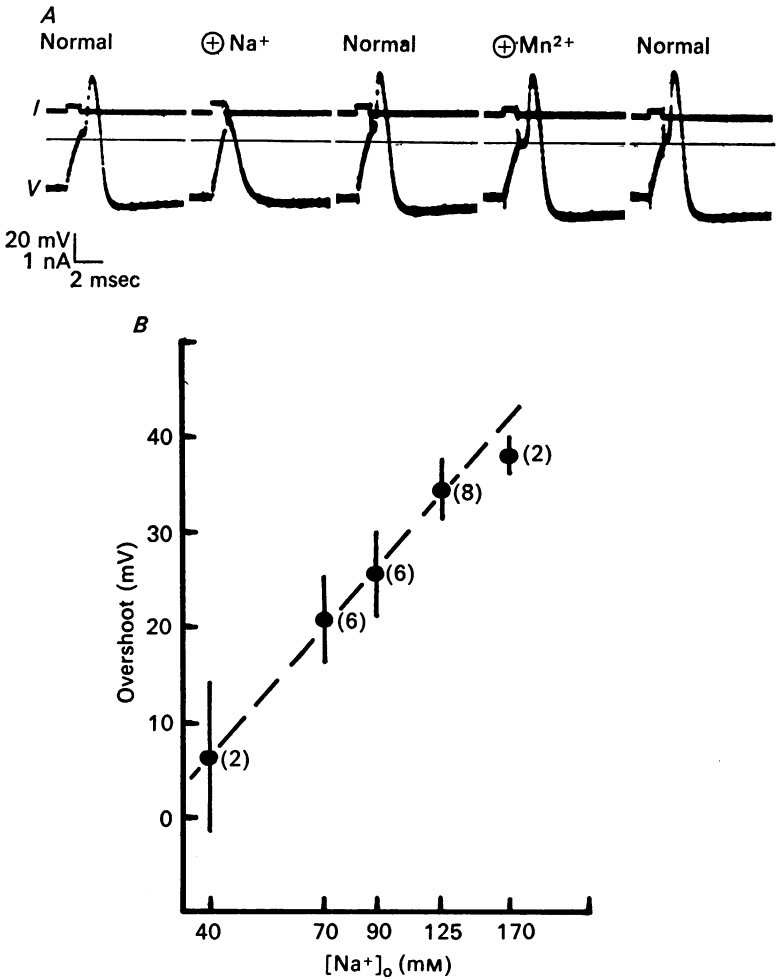

Fig. 8. Ionic dependence of action potentials at late stages. *A*: the action potential in a cell is abolished by removal of Na^+ (note larger current pulse) and largely unaffected by the addition of Mn^{2+}; Stage 44. *B*: the overshoot is plotted as a function of the logarithm of the external $[Na^+]$. Numbers in parentheses indicate the number of cells from which the mean and standard error were calculated. The least squares fit to the points has a slope of 57·4 mV/decade change in $[Na^+]_o$. Stages 47–49.

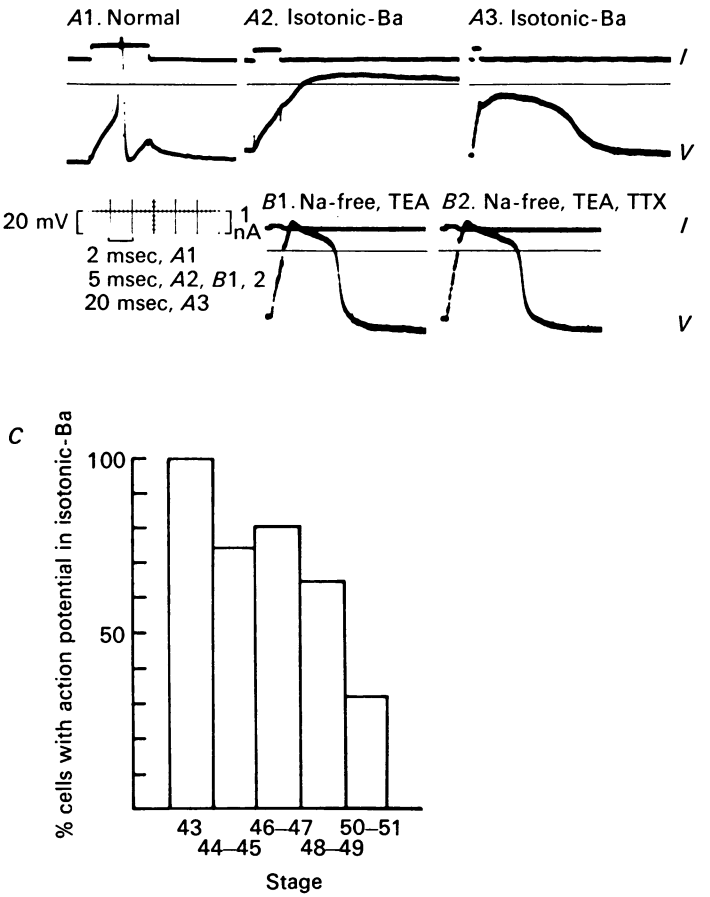

Fig. 9. Ba^{2+} and Ca^{2+} dependent action potentials in cells at late stages. *A1*: normal action potential. *A2*: initial response of the same cell in isotonic-Ba solution (Na-free). *A3*: the fourth action potential in the same cell in isotonic-Ba (only one further regenerative response was obtained); stage 48. *B*: action potential in another cell in Na-free solution plus 60 mM-TEA (*B1*), plus TTX (*B2*); stage 48/49. *C*: percentage of cells capable of these long duration action potentials in isotonic-Ba, as a function of developmental age; 150 cells tested. By stages 50–51, two-thirds of cells have lost this capability.

these cells while they were perfused with Na-free solutions in which the outward current channel (g_K) was blocked. Many cells with conventional action potentials of approx. 1 msec duration were found to produce regenerative responses in isotonic-Ba solution (Fig. 9*A*). The duration of these responses declined abruptly at first, and then more gradually. Mean durations were 7·9, 3·1, 2·8 and 2·0 sec, for the first four responses,

respectively. The overshoot declined significantly only after the first response, mean values for the first four responses being 55, 39, 37 and 38 mV. Often the cells produced regenerative responses only once or twice before becoming inexcitable. These responses were unaffected by TTX, but blocked by La^{3+} or Co^{2+}, suggesting that the inward current is flowing through divalent cation channels. Upon return to normal saline, the cells again fired typical Na-dependent action potentials. The percentage capable of producing action potentials in isotonic-Ba decreased from 100 % at stage 43, to 33 % at stages 50–51 (Fig. 9*C*), although all cells were able to produce Na-dependent action potentials in normal saline.

A smaller number of cells was examined in Na-free solution plus TEA, and observed to produce a regenerative response of long duration (Fig. 9*B*). However, neither its duration nor overshoot decreased appreciably after the first response. The concentration of the divalent cation in these experiments (10 mM-Ca^{2+}) was much less than in isotonic-Ba solutions (110 mM-Ba^{2+}). As in the experiments described in the previous paragraph, the percentage of cells capable of producing these action potentials decreased with increasing developmental age.

Time course of development

During the period of development studied here the duration of the action potential has diminished from a maximum of 1100 to 1·5 msec (Fig. 10). There is considerable variability in the duration of action potentials recorded at early stages, although all depend on an inward Ca^{2+} current. This variability occurs from animal to animal at the same morphological stage, and even within one animal. No consistent antero-posterior gradient has been detected. The variability of the duration of the action potential may be related to the number of times the cell has fired prior to initial examination. The input resistance of the cells has fallen from a max. of 1000–120 MΩ, following a similar time course (Fig. 10). The diameter of the cell body does not change appreciably during this time (Spitzer & Baccaglini, 1976). The stages at which g_{Ca} and g_{Na} are usually first detected have been indicated. The later loss of g_{Ca} occurs over a period of 2 weeks.

DISCUSSION

Inward currents in Rohon-Beard neurones

The ionic basis of the action potential of Rohon-Beard neurones changes during development, over a period of 3–4 days. The cells are initially inexcitable. There are four lines of evidence that Ca^{2+} is largely responsible for the inward current, when an action potential can first be elicited at the time of closure of the neural tube. The first is the ion selectivity of the

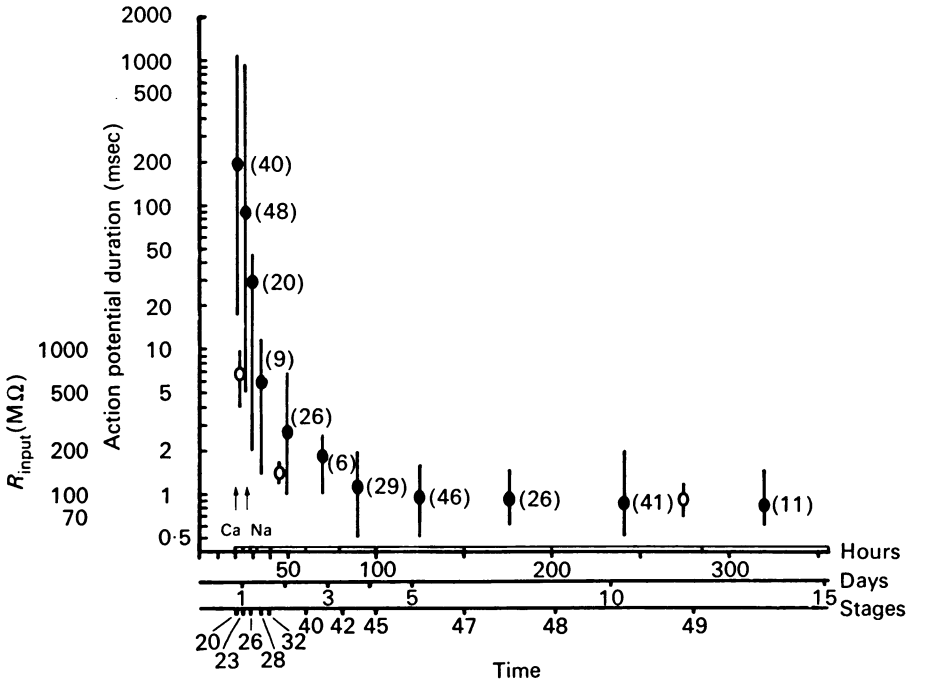

Fig. 10. Duration of action potential and input resistance of cells as functions of age, indicated in hours, days, and stages. Duration (filled circles): the number of cells from which the value of mean and range were calculated is indicated in parentheses for each time period shown on the abscissa. Values decline rapidly (note logarithmic scale), over more than two orders of magnitude and then level off. Variability is initially large. Input resistance (open circles): values decrease during development. The changes are not as great as those in duration of the action potential. The times at which inward Ca^{2+} and Na^{+} currents first appear are indicated by arrows.

response. Removal of either Na^{+} or Cl^{-} does not change the amplitude of the action potential. The conductance measurements indicate that the major conductance increase at the beginning of the plateau is due neither to Na^{+} nor to K^{+}, since removal of Na^{+} and addition of TEA do not alter the values. Ca^{2+} is the remaining ion in normal saline distributed in an appropriate concentration gradient to produce the observed response. Previous results also indicated that the overshoot of the action potential varies linearly with the log of the external Ca^{2+} concentration, as predicted by the Nernst equation (Spitzer & Baccaglini, 1976). Although the magnitude of the overshoot varies from cell to cell, in any one cell the overshoot shows the same dependence on $[Ca^{2+}]_o$. In the same study, Ba^{2+} was shown to substitute for Ca^{2+} at these stages in development.

A second line of evidence is the pharmacology of the response. The

action potential is blocked by La^{3+}, Co^{2+} and Mn^{2+}, but unaffected by TTX. The toxin has been shown to block Na^+ but not Ca^{2+} channels in mature excitable cells (Baker, Meves & Ridgeway, 1971; Hagiwara & Nakajima, 1966).

A third line of evidence is the agreement between the measured and calculated values of the calcium conductance. The mean of the experimental values determined for eleven cells by square pulse analysis is $3{\cdot}1 \pm 1{\cdot}0 \times 10^{-9}$ mho (see above). The value of g_{Ca} can be calculated if the cells at the peak of the action potential have only two parallel conductances, g_{Ca} and the resting g_{RP}, each in series with the equilibrium potentials E_{Ca} and E_{RP} (Katz & Miledi, 1969). Under these conditions,

$$g_{Ca} = g_{RP} \frac{(E - E_{RP})}{(E_{Ca} - E)}, \quad E = \text{overshoot}.$$

The overshoot was measured for each of the same eleven cells, and the resting conductance determined from the values of input resistance. E_{RP} is taken as -88 mV (Spitzer, 1976). If $[Ca^{2+}]_i = 10^{-7}$ M, which is a reasonable assumption (Baker & Warner, 1972), $E_{Ca} = +145$ mV. The mean Ca^{2+} conductance calculated in this manner for these cells is $3{\cdot}1 \pm 1{\cdot}2 \times 10^{-9}$ mho. Values of E_{RP} are often smaller than -88 mV, which would reduce the calculated value of g_{Ca}. However, this agreement suggests that the increase in conductance during the action potential is due largely to Ca^{2+}.

The fourth line of evidence is the ability of the cells to produce action potentials at low resting potentials. This indicates that the inward current channel is not inactivated until the membrane voltage is close to zero, a feature common to other Ca^{2+} channels (Rougier *et al.* 1969; Mascher & Peper, 1969; Horackova & Vassort, 1976; Miyazaki *et al.* 1974*b*).

At intermediate stages of development, the inward current is carried by both Na^+ and Ca^{2+}. The rapidly rising spike portion of the action potential can be eliminated by the removal of Na^+ or addition of TTX, leaving a slower plateau component which resembles that due to Ca^{2+} influx at earlier stages. In contrast, the Ca^{2+} component is eliminated by La^{3+}, Co^{2+} and Mn^{2+}, leaving the Na^+ spike.

At late stages, the inward current is carried chiefly by Na^+ in normal saline, as shown by the following three lines of evidence. First, the ion selectivity is demonstrated by the linear relationship between the overshoot and the log of $[Na^+]_o$, which follows closely the prediction of the Nernst equation for a Na^+ electrode. Secondly, the pharmacology of the response indicates the role of g_{Na}, since it is blocked by TTX and not greatly affected by La^{3+}, Co^{2+}, or Mn^{2+}. Thirdly, in many cells it is not possible to elicit a regenerative response in Na-free solutions, in which divalent ions are available to carry inward current, even in the presence of

agents which block the outward current channel. The apparent disappearance of the calcium conductance could occur in several ways (see later Discussion). We have not excluded the possibility that some g_K becomes resistant to both TEA and isotonic-Ba, so that a residual outward current masks the presence of the inward calcium current.

Cultures of cells dissociated from *Xenopus* neural plate at stage 15 yield nerve cells that appear to go through this developmental sequence *in vitro* The ionic dependence of the inward current of the action potential seems to go through the same changes on the same time course seen *in vivo* (Spitzer & Lamborghini, 1976).

There is some variability in the precise timing of changes in the ionic basis and the duration of the action potential. It is clear from these studies *in vivo* that Rohon-Beard neurones are not a completely synchronously developing population. Similar variability has been reported for the less homogeneous group of neurones in culture. The timing of the initial expression of g_{Ca} and g_{Na} varies by several hours. This finding might be due to small variations in the time of induction of the cells, or the rate of subsequent development. The duration of the action potential changes during development, along with its ionic dependence; this process seems continuously graded.

Inward currents in other developing cells

The initial electrical inexcitability of Rohon-Beard cells is consistent with the previous results from other cells of amphibian embryos at early stages. The egg and blastula cells of *Xenopus* have been found incapable of producing a regenerative response (Palmer & Slack, 1970; Slack & Warner, 1975). Similar observations have been made on presumptive neural cells of *Ambystoma* at mid-neural fold stages (Warner, 1973). In contrast, it has been demonstrated that some invertebrate eggs are capable of making action potentials (Miyazaki *et al.* 1972, 1974*a*, 1975; Hagiwara *et al.* 1975; Shen & Steinhardt, 1976).

A sequence of changes in excitability like those in Rohon-Beard neurones occurs in developing chick muscle cells *in vivo* (Kano, 1975). These cells are also inexcitable at first. When a regenerative response can be elicited, the ions carrying most of the inward current change from Ca^{2+}, to Na^+ and Ca^{2+} to Na^+. This response also decreases in duration; the whole process takes 7–15 days. The same variety of electrical responses was observed in chick muscle *in vitro*, although the type of action potential has not yet been correlated with the age of the culture (Kano & Shimada, 1973).

Somewhat different developmental changes have been found in other systems. In the embryogenesis of the tunicate, mosaic development permits observation of the presumptive muscle cells from early stages. The ionic

dependence of the action potential shifts from Na^+ and Ca^{2+}, in the egg, to Ca^{2+} in the mature muscle (Takahashi *et al.* 1971; Miyazaki *et al.* 1972). In the development of the chick, much of the inward current of the action potential in aggregates of embryonic cardiac muscle cells is initially carried by Ca^{2+}; a prominent Na^+ component appears at later times (McDonald & Sachs, 1975). The TTX-resistant Na^+ channel in chick cardiac muscle *in vivo* becomes sensitive to TTX later in development (Sperelakis & Shigenobu, 1972). In a clonal line of rat skeletal muscle cells (L6) in culture the inward current is carried first by Na^+, and later by both Na^+ and Ca^{2+} (Kidokoro, 1973; 1975*a*, *b*).

Calcium entering the neurones during the action potential

It is possible to calculate the calcium current that exists at the onset of the plateau of the action potential in these experiments at stages 20–25, from $i_{Ca} = g_{Ca}\ (E - E_{Ca})$. Using the values for g_{Ca} from the eleven cells discussed above, E_{Ca} as previously, and the mean overshoot of +24 mV, $i_{Ca} = 3{\cdot}8 \times 10^{-10}$ A. The value of the inward current can also be calculated from $i_{Ca} = -C\,dV/dt$, when dV/dt is a maximum (Hodgkin & Katz, 1949). The cell in Fig. 4 has a max. rate of rise of slightly more than 3 V/sec; determination of the value of C from $\tau = RC$ gives a current of $4{\cdot}0 \times 10^{-10}$A, in reasonable agreement with the calculations from the conductance measurements. In order to obtain a rough estimate of the amount of Ca^{2+} entering the cell per action potential, we assume that $g_{Ca}\,(t)$ = constant = 10^{-9} mho, and take a mean duration of 200 msec. $Q_{Ca} = i_{Ca} \times t = 2{\cdot}4 \times 10^{-11} = 1{\cdot}2 \times 10^{-16}$ moles $= 0{\cdot}7 \times 10^8$ ions/impulse. Assuming as previously, that cells are 20 μm spheres with negligible processes, this quantity of Ca^{2+} could raise the internal concentration from 10^{-7} to $2{\cdot}8 \times 10^{-5}$ M. Such an increase would be expected to change E_{Ca}; since the overshoot does not change during repetitive stimulation, Ca^{2+} must rapidly be sequestered intracellularly. The extracellular concentration of Ca^{2+} in intact *Xenopus* neurulae is not known (however, see Slack, Warner & Warren, 1973), and there is no information about the frequency of action potentials occurring *in vivo*. However, even a single action potential in lower $[Ca^{2+}]_o$ would cause an influx of substantial amounts of Ca^{2+}.

g_{Ca} and I_{Ca} are $2{\cdot}6 \times 10^{-4}$ mho/cm^2 and $3{\cdot}1 \times 10^{-5}$ A/cm^2, assuming the surface membrane area to be the same as above. These values are somewhat smaller than those found for several adult cells: the presynaptic terminal of the giant synapse of squid, 10^{-3} mho/cm^2, 10^{-4} A/cm^2 (Katz & Miledi, 1969); barnacle muscle fibre, $0{\cdot}7 \times 10^{-3}$ mho/cm^2 (Keynes, Rojas, Taylor & Vergara, 1973), 10^{-4} A/cm^2 (Hagiwara *et al.* 1969). They are much larger than those observed in amphibian slow muscle fibres: $1{\cdot}3 \times 10^{-6}$ mho/cm^2, $1{\cdot}8 \times 10^{-7}$ A/cm^2 (Stefani & Uchitel, 1976), but close to the values for amphibian

twitch muscle fibres: $1{\cdot}2 \times 10^{-4}$ mho/cm^2, 10^{-5} A/cm^2 (Beaty & Stefani, 1976). The rate of decay of g_{Ca} in Rohon-Beard neurones is similar to that seen in the presynaptic terminal of the squid, and considerably less than that in the slow muscle fibres (half-life = 6·8 sec). In developing systems Hagiwara *et al.* (1975) have found I_{Ca} of 3·3 and $3{\cdot}5 \times 10^{-6}$ A/cm^2 in starfish eggs. A value of 10^{-5} A/cm^2 has been reported for a different species of the same animal in $10 \times$ Ca solutions (Miyazaki *et al.* 1975). Developing tunicate eggs have I_{Ca} of $< 2 \times 10^{-6}$ A/cm^2, in high Ca^{2+} solutions (Miyazaki *et al.* 1975; Okamoto, Takahashi & Yoshii, 1976).

Changes in the duration of the early action potential in single cells

The shortening of the calcium action potential with repetitive stimulation at early stages is similar to the observations of Katz & Miledi (1969). The regenerative response elicited from the giant presynaptic terminal in the squid under special conditions was found to shorten during stimulation at 0·4 Hz. The overshoot of the action potential in Rohon-Beard cells remains constant in normal saline. In contrast, the overshoot in isotonic-Ba in cells at late stages declines abruptly after the first action potential. This is consistent with a decline in the divalent cation concentration gradient and suggests that the cells cannot sequester large amounts of Ba^{2+} in the same way as the smaller amount of Ca^{2+}. The process of shortening the action potential in Rohon-Beard cells may be attributable to a decrease in the time required to activate a g_K rather than to the inactivation of g_{Ca} (see Katz & Miledi, 1971). The inactivation of g_{Ca} in barnacle muscle has been shown to involve elevation of the intracellular free Ca^{2+} levels (Hagiwara & Nakajima, 1966), a condition that seems not to occur in Rohon-Beard cells. The influx of calcium has been shown to increase g_K transiently in invertebrate neurones (Meech & Strumwasser, 1970; Meech, 1974; Meech & Standen, 1975) and in spinal motoneurones (Krnjević & Lisiewicz, 1972). A Ca-dependent, TEA-insensitive g_K has been reported for frog motoneurones (Barrett & Barrett, 1976). The increased bound intracellular calcium in Rohon-Beard neurones may be involved in a slower process of maturation of g_K, which can then be activated to bring about the earlier termination of the action potential. The repolarization at late stages is not, however, affected acutely by agents that block calcium influx.

Outward current

In contrast to the changes seen in the ionic basis of the inward current during the early stages of development of *Xenopus* described here, no qualitative change was observed in the ionic basis of the outward current. The outward current channel exists from the time that the Ca^{2+} channel is detected in the membrane. This is shown by the rapid repolarization and

hyperpolarization at the termination of the action potential, at stages when it is first elicited. The repolarization is also slowed and the hyperpolarization reduced in the presence of TEA, which prolongs the action potential. Koppenhöfer (1967) has shown that 5 mM TEA reduces potassium currents to almost zero at *Xenopus* nodes of Ranvier. Our results are consistent with the observation that delayed rectification is present in the membrane of neural plate cells of *Ambystoma* embryos at a time when no action potential can be obtained (Warner, 1973). Similar conclusions may be drawn from the observations on chick muscle fibres (Kano, 1975), which reveal a hyperpolarization following the repolarization of the earliest recorded action potentials. The outward current channel appears before or with the inward current channel in the development of both systems. In contrast, marked delayed rectification appears some time after the inward current channel in developing tunicate muscle cells (Takahashi *et al.* 1971).

Function of the calcium action potential

What may be the role of an action potential in which the inward current is carried by calcium, in the development of excitable cells? One possibility is that it is important for the signalling function of the cells. There is some evidence that the Na^+ concentration gradient across cell membranes in *Xenopus* embryos is less than that in adults, because of large intracellular values (Slack, Warner & Warren, 1973; Slack & Warner, 1975); the driving force on Na^+ may be insufficient to produce action potentials. Another possibility is that it provides a way to deliver Ca^{2+} to the inside of the cell, in the correct amount and with the appropriate localization, to promote some aspect of cellular development. It may be that intracellular calcium levels must be raised to activate the mechanism that causes progressive shortening of the action potential, perhaps g_K. Alternatively, intracellular Ca^{2+} may be implicated in the movement of filaments often associated with the extension of cell processes (reviewed by Bray & Bunge, 1973), or the synthesis of cyclic nucleotides (cGMP; Schultz *et al.* 1973). The Rohon-Beard cells die during development of the tadpole; it is not known if there is a connexion between changes in excitability and cell death.

Developmental mechanisms

At present it is only possible to speculate about the mechanisms underlying this developmental sequence. They could involve the synthesis of macromolecules (e.g. channel proteins) and their insertion in the membrane during a brief period in the development of the cells. These molecules might then become degraded or removed from the membrane over a longer period. On the other hand, it could be that all the Ca^{2+} and Na^+ channels exist in the membrane at the early stages, and are activated and

later inactivated by a change in covalent linkage (cleavage of small peptide) or a conformational change resulting from alterations in cytoplasmic composition. Whether Ca^{2+} and Na^{+} are carried by separate channels, or by modification of one channel, is a related question that is presently unanswered. The observation of this developmental sequence in isolated neurones in cell culture may afford an opportunity to distinguish between these alternatives.

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