RNA synthesis dependence of action potential development in spinal cord neurones

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The role of transcription in the development of electrical properties of neuronal membranes has been largely unexplored. To study the molecular events which result in the expression of these properties it is useful to describe the timing of the underlying RNA synthetic events. For example, the timing of the transcription involved in denervation-induced action potentials in frog slow muscle fibres[1] and brain extract-induced sodium channels in chick muscle cells[2] has been investigated. Previous studies of *Xenopus laevis* spinal neurones have established that the timing of the development of the neuronal action potential ionic dependence in dissociated cell cultures parallels that seen *in vivo*[3-6]. This culture system, therefore, allows the determination of transcription-dependent periods necessary for the development of membrane properties known to have *in vivo* relevance. In the study described here, actinomycin D was used to examine the timing of the RNA synthetic events necessary for (1) neurite outgrowth and (2) development of the ionic dependence of the action potential. I report that inhibition of transcription at an early stage specifically blocks the appearance of the mature sodium-dependent action potential without affecting either neurite outgrowth or the development of delayed rectification.

Many cell types differentiate in dissociated cell cultures prepared from neural plate-stage *Xenopus* embryos; neurones are recognized only after the extension of neurites, which begins ~6 h after plating. When actinomycin D, which inhibits DNA-directed RNA synthesis[7], at 2.5 $\mu g\ ml^{-1}$ was added to the cultures earlier than 1.5 h after plating (19 h after fertilization), there was no obvious morphological differentiation of any cell type. However, chronic exposure to actinomycin D begun between 1.5 and 4 h after plating inhibited neuronal incorporation of ^{3}H-uridine (Fig. 1) but allowed extension of neurites from cells at ~6 h, as observed for untreated cells. Over the next 2.5 days the range of morphological variability seen in neurites from actinomycin D-treated neurones was similar to that in control cells, although there were fewer neurones in the inhibited cultures (a reduction in the range of 20 to 80%). There were also fewer differentiated cells of other types in the actinomycin D-treated cultures.

To determine whether the effect of actinomycin D on neurite outgrowth was stage-dependent, the inhibitor was added at various times after plating to cultures made from embryos at stages between the late gastrula and late neurula. Actinomycin D could be added at progressively earlier times after plating, as cultures were prepared from later stage embryos, and still allow neurite extension. For example, late neurula embryos corresponding to 19 h after fertilization were cultured directly into actinomycin D and still extended neurites at times comparable to controls. Thus the RNAs necessary for neuronal survival or neurite elaboration seem to be transcribed before 1.5 h after culturing, that is, before 19 h after fertilization. In PC12 cells, initiation of *de novo* neurite outgrowth also requires transcription before RNA synthesis-independent growth can proceed[8]. The experiments reported here do not exclude the possibility that neurones do not survive actinomycin D treatment begun prior to 19 h after fertilization. However, use of reversible metabolic inhibitors for protein synthesis in *Xenopus* neurones[9] and for RNA synthesis in PC12 cells[10] suggests that it is possible for cultured cells to survive periods of synthesis inhibition, and initiate or resume neurite outgrowth on resumption of synthesis.

The duration of the action potential is an indication of its ionic dependence, and thus the developmental maturity of these spinal cord neurones[4]. Young cells from control cultures have action potentials of long and variable duration which are primarily calcium-dependent. At slightly later times the action potential duration decreases and an overshooting sodium component and a small calcium component are present. Late-stage action potentials are very brief and primarily sodium-dependent. Figure 2 illustrates the action potential duration as a function of age in culture. The durations seen in control neurones declined whereas the action potentials of the actinomycin D-treated neurones examined over the same time period did not decrease in duration or variability. These data suggest that actinomycin D-treated neurones maintain a primarily calcium-dependent action potential even at late times in culture.

The ionic dependence of the action potentials was examined directly by ionic substitutions and addition of blocking agents to the perfusion saline. At early times in culture both control and actinomycin D-treated neurones exhibited overshooting action potentials that were primarily calcium-dependent events with a slow sodium component (Fig. 3*A*, *B*). Control neurones examined at 50 h in culture showed short-duration sodium-dependent action potentials (Fig. 3*C*). In contrast, the long-duration action potentials of the inhibited neurones were primarily calcium-dependent, even at 50 h in culture. The sodium component at this time appeared unchanged from that seen in control and experimental neurones at early times (Fig. 3*D*). Thus, the development of the ionic dependence of the

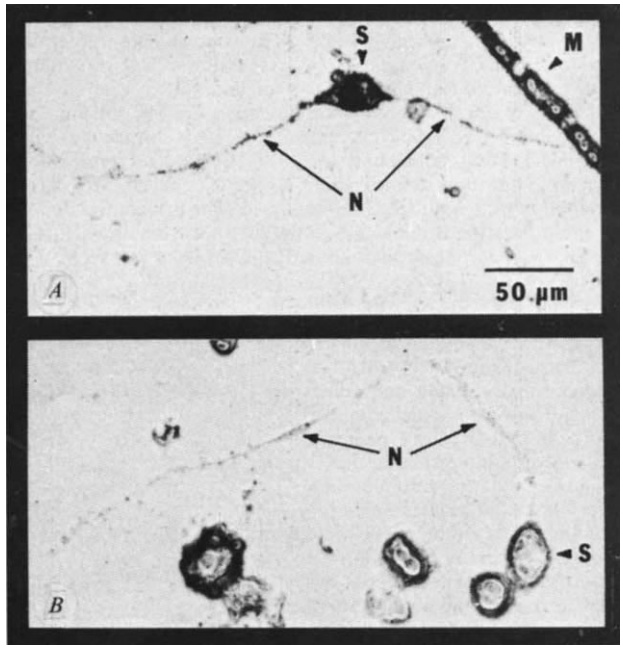

Fig. 1 Neuronal morphology and incorporation of ^{3}H-uridine in control and actinomycin D-treated cultures. *A*, Control neurone viewed by brightfield illumination at 24 h *in vitro* shows dense labelling of the soma (S), and neurites (N). *B*, A neurone from an actinomycin D-treated culture is morphologically similar to the control at 24 h, but shows no radioactive labelling above background. As the number of neurones does not increase after 24 h and actinomycin D is chronically present, it is likely that neurones examined physiologically at times after 24 h exhibit comparable levels of inhibition. Dissociated cell cultures were prepared from neural plate-stage embryos[4,14], in a simple, fully defined medium[9]. To determine the level of inhibition by actinomycin D, control cultures were exposed to 5 $\mu Ci\ ml^{-1}$ ^{3}H-uridine from 2 to 24 h, at which time they were fixed and processed for autoradiography. Experimental cultures were prepared similarly, with the addition of 2.5 $\mu g\ ml^{-1}$ actinomycin D 15 min before exposure to ^{3}H-uridine. Actinomycin D at 2.5 $\mu g\ ml^{-1}$ also inhibited >95% of the incorporation of ^{3}H-uridine into the TCA-precipitable cell fraction from these cultures.

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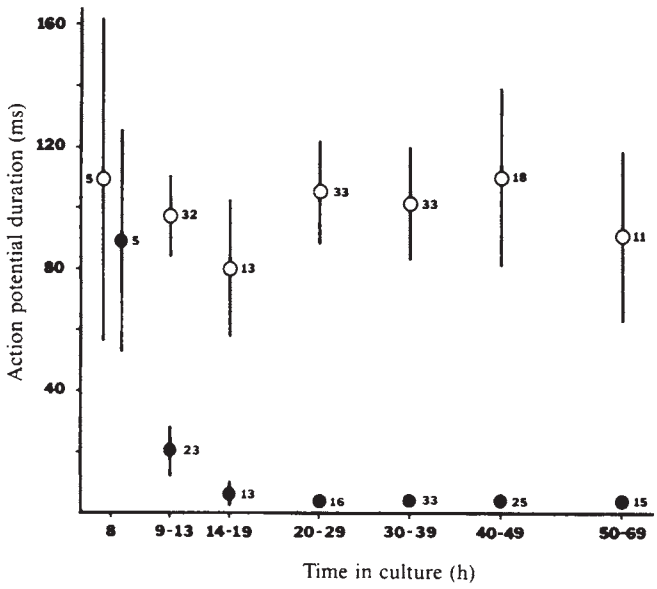

Fig. 2 The action potential duration as a function of time in culture. Action potentials were elicited in response to short depolarizing current pulses in neurones between 8 and 69 h after plating. Each point was determined by averaging action potential durations in normal saline (NaCl, 40 mM; $CaCl_2$, 10 mM; HEPES, 5 mM (*p*H 7.4); KCl, 3 mM) for the number of neurones indicated next to each point on the graph; bars indicate s.e.m. The resting potential of both control and actinomycin D-treated neurones ranged between −50 and −90 mV. The control action potential duration (filled circles) at 8 h is long and exhibits a large variability. Both the duration and variability decrease with increasing age, indicating a developmental switch from primarily calcium to sodium ionic dependence. Neurones treated chronically with actinomycin D first added between 19 and 21.5 h after fertilization have long-duration action potentials (open circles) with large variability throughout the time examined. The 8-h control action potential duration is not significantly different from any of those seen in neurones from inhibited cultures from 8 to 69 h.

action potential of the inhibited neurones seems to have been arrested at an early developmental stage. Selective survival cannot account for these results as none of the untreated neurones at late times showed long-duration calcium-dependent action potentials. These results suggest that the RNAs necessary for acquisition and maintenance of a calcium action potential with an early sodium component are synthesized earlier than 19 h after fertilization but that the maturation of the fast sodium component requires a period of RNA synthesis that extends beyond 19 h.

The persistence of long-duration action potentials of mixed ionic dependence seen in inhibited neurones could be explained by the effect of RNA synthesis inhibition on any of several developing properties of the neurones. Inhibition may prevent both an increase in sodium conductance during development and simultaneously a decrease in calcium conductance. Another possibility is that actinomycin D affects the development of outward currents involved in repolarization of the action potential. To investigate this last possibility, delayed rectification was examined. A voltage-dependent conductance increase yielding delayed rectification was present at 7–10 h and increased by ~50% between 10 and 13 h in both control and experimental cultures (Table 1). These results indicate that the development of delayed rectification is not grossly affected by the presence of actinomycin D, and that the increase in voltage-dependent delayed rectification does not have a major role in determining the duration of the action potential in actinomycin D-treated neurones. The input resistance seems neither to change over the time period examined nor to be altered by the presence of actinomycin D.

Does the RNA synthesis inhibition of the impulse show a stage dependence similar to that seen for neurite outgrowth? To address this question, inhibition of RNA synthesis was initiated at progressively later times between 21.5 and 35 h after fertilization. This allowed development of neurones having progressively more mature action potentials, an increasing percentage of the inward current carried by sodium and a decreasing calcium component. Thus, the development of the impulse requires a period of RNA synthesis that extends for 30–35 h after fertilization and it seems to be affected in a stage-depen-

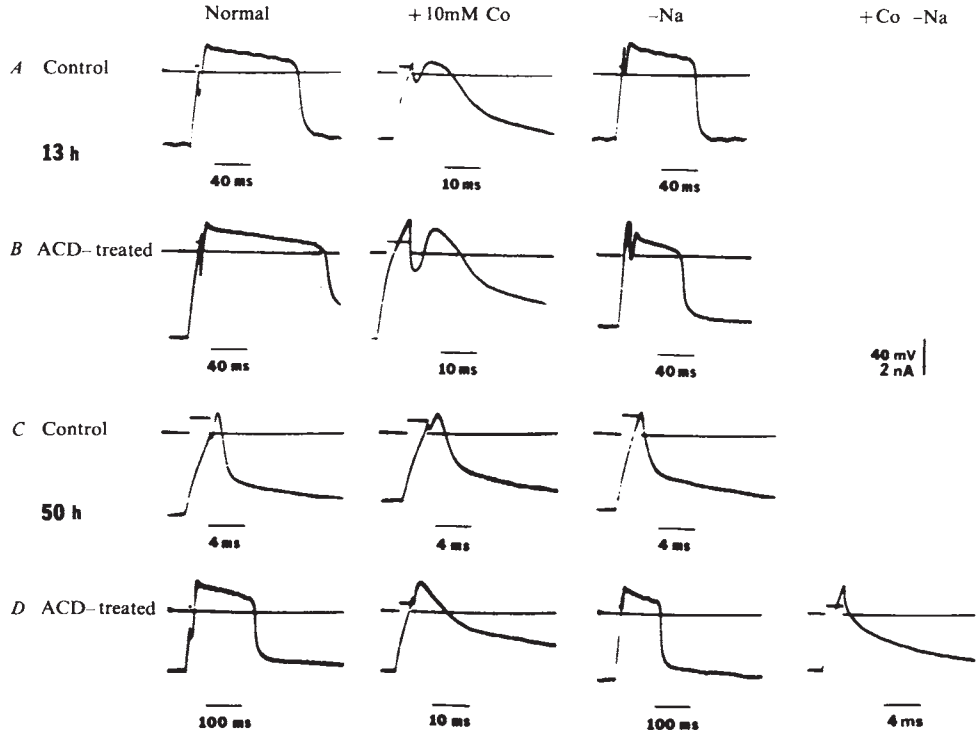

Fig. 3 Ionic dependence of action potentials from control and actinomycin D-treated neurones at early and late times in development; records from four cells. Upper trace in all records indicates the current amplitude and the zero potential; lower trace indicates membrane potential. Time calibration is noted below each trace. *A* and *B* illustrate action potentials of neurones at early times in control and actinomycin D (ACD)-treated cultures (13 h) which have a long-duration, overshooting calcium component that is blocked by cobalt. A slow sodium component can be detected in the presence of cobalt. *C* Shows a mature sodium-dependent action potential from a control neurone at 50 h. The short-duration action potential is present in normal saline and in saline plus cobalt but cannot be elicited when external sodium is removed. In contrast, the action potential of the actinomycin D-treated neurone at 50 h (*D*) is still primarily calcium-dependent, similar to the action potential from both control and actinomycin D-treated neurones at early times (*A* and *B*), with a slow sodium component that is present in cobalt but is blocked on removal of sodium. Accurate determination of the voltage necessary for impulse initiation was not possible from these records in which current was passed and voltage recorded with the same electrode.

Table 1 Rectifying conductance develops similarly in neurones from control and experimental cultures

Hours in culture	Rectifying conductance (%) Control	Actinomycin D-treated
7–10	0.39 ± 0.02 (7)	0.33 ± 0.06 (8)
13–15	0.53 ± 0.10 (5)	0.58 ± 0.04 (6)
19–21	0.49 ± 0.05 (8)	0.50 ± 0.06 (10)
29–31	0.50 ± 0.05 (4)	0.53 ± 0.08 (5)

Values are mean ± s.e.m. (n). Current–voltage curves were constructed by measuring the voltage response of the membrane to long current pulses (800 ms) of small amplitude (−10 to +20 pA) with a single electrode that was in balance before and after the measurements were made. The outward currents activated within 30 ms and did not inactivate within 800 ms. An increase in conductance due to activation of the late outward current channels was seen in both control and actinomycin D-treated neurones between 10 and 13 h, indicating the normal development of delayed rectification in the presence of actinomycin D. The per cent conductance due to delayed rectification (rectifying conductance, %) is defined as $G_R - G_P/G_R$, where G_P is the slope of the I–V plot in the hyperpolarizing quadrant and G_R (conductance after rectification) is the slope of the best fit line in the depolarizing quadrant of the points that deviate from G_P. Inward currents were suppressed pharmacologically with 10^{-6} g ml^{-1} tetrodotoxin and 10 mM $CoCl_2$. The conductances measured in actinomycin D-treated and control neurones at 7–10 h are not significantly different (Student's t-test). Experimental and control conductances between 13 and 31 h also fail the significance test. However, experimental and control conductances at 7–10 h are significantly different from the pooled conductances at later times.

dent manner by actinomycin D. Although the possibility has not been excluded, it seems unlikely for two reasons that actinomycin D affects nontranscriptional events, for example, inhibition of DNA and protein synthesis: (1) Blair has shown[9] that direct inhibition of protein synthesis affects the same properties reported here with a stage dependency 3 h later in development; (2) the cultures were treated with a relatively low concentration of actinomycin D[11].

Two of the properties examined—neurite outgrowth and the development of delayed rectification—seem to mature normally in these cells during inhibition of RNA synthesis begun 2–4.5 h before neurite outgrowth takes place. This finding is consistent with the observations that morphological differentiation of *Drosophila* and murine superior cervical ganglion neurones requires transcription before the first axons are seen, after which time the neurones enter an actinomycin D-insensitive period during which axon elongation proceeds[12,13]. Two other properties, the resting potential and imput resistance, also appear unaltered in the *Xenopus* neurones exposed to actinomycin D. In contrast, maturation of the fast sodium action potential apparently requires an extended period of RNA synthesis. The finding that the calcium action potential is similar at both 8 and 69 h during exposure to actinomycin D raises the possibility that the metabolic turnover of RNAs and proteins responsible for maintenance of this phenotype is rather slow. Examination of RNA synthesis-dependent periods in simple developing systems such as this one may provide an understanding of the molecular mechanisms by which the early programme of differentiation is elaborated.

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Transmitter-like action of ATP on patched membranes of cultured myoblasts and myotubes

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The concept of purinergic neurotransmission, first proposed by Burnstock[1], has been confirmed in various cell types. We show here, by the patch-clamp method[2], that external ATP in micromolar concentrations (1–100 μM) activates cation channels in the membranes of fusion-competent myoblasts and myotubes. In cell-attached membrane patches of myoblasts and myotubes the mean number of simultaneously activated channels increases with time after external ATP application. In myoblasts only one population of channels having a mean single-channel conductance of $\gamma = 43$ pS was found, while in myotubes two populations with $\gamma_1 = 48$ pS and $\gamma_2 = 20$ pS were observed. Treatment of myotube membranes with acetylcholine (ACh) or carbachol resulted in two populations of channels which had conductance values and voltage-dependent mean channel lifetimes similar to those produced in response to ATP. The results show that embryonic skeletal muscle cells contain cation channels sensitive to ATP and provide evidence for a neurotransmitter-like action of ATP on these cells.

Myoblasts were prepared from 12-day-old chicken embryos and cultured as previously described[3]. The cells were maintained in a medium Ca^{2+} concentration of 10^{-7} M for 48 h, during which time they achieved fusion competence; raising the Ca^{2+} concentration at this time to 1.4 mM results in rapid, synchronous fusion. Thirty minutes before measurements the essentially Ca^{2+}-free culture medium was replaced with the following solution (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.4, $MgCl_2$ 0.8, HEPES 10, pH 7.4, glucose 10. The Ca^{2+} concentration was increased at this point to allow measurements in more physiological conditions and to improve the pipette–cell attachment; Ca^{2+}-free solutions gave similar results. Pipettes used for recording single-channel currents were filled with solutions containing ATP, ACh or carbachol. Experiments were performed at 20–22 °C.

In myoblasts, addition of 1–100 μM ATP to the pipette solution resulted in the induction of single elementary current events of similar amplitudes (Fig. 1). At ATP concentrations >10 μM, the mean number of channels activated simultaneously increased visibly with time (Fig. 1*A*, *B*). From the linear current–voltage relationship in the voltage range between the resting potential (V_m) and $V_m - 120$ mV, a zero-current potential of $V_m + 38 \pm 6$ mV (mean ± s.e.; $n = 6$) was derived by extrapolation. The single-channel conductance, γ, was 43 ± 3 pS ($n = 6$). The channel showed no detectable selectivity between Na^+ and K^+, it did not seem to be significantly permeable to Ca^{2+} and was not blocked by raising the extracellular Ca^{2+} concentration to 16.4 mM (Fig. 2*A*). Figure 2 also shows that the mean current in the presence of ATP increased with hyperpolarizing membrane potential as a result of several voltage-dependent processes. First, there was an increase in single-channel current, due to its ohmic behaviour (straight lower line in current record). Second, there was a potential-dependent

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