Stimulation of DNA Synthesis in CNS Neurones by Sustained Depolarisation

IT has been proposed[1,2] that intracellular ionic concentration levels associated with the electrical transmembrane potential (E_m) of somatic cells may be functionally involved in mitogenesis control, cells possessing a low E_m level (−10 to −20 mV) being mitogenically active. This hypothesis has been supported by subsequent demonstrations that imposed intracellular ionic conditions simulating high E_m levels effectively but reversibly block mitogenesis[3,4]. As a corollary of this hypothesis, it was suggested[2] that mitogenesis might be activated in highly polarised non-dividing cells (such as mature CNS neurones and muscle) by maintaining them in a sufficiently depolarised state. We have investigated this possibility for the case of chick spinal cord neurones depolarised with the cardiac glycoside ouabain.

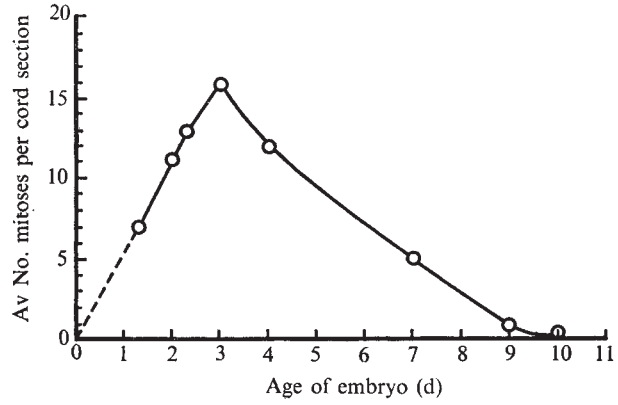

Fig. 1 Variation with time of mitotic activity in chick spinal cord neurones *in ovo*. Each data point represents the average number of mitotic cells (visible chromosomes) per cord section; thirty sections per cord, and one cord per developmental stage were examined.

Cells obtained from trypsin-dissociated spinal cords of 7–10 d chick embryos were cultured after the method of Fischbach[5] on collagen-coated glass coverslips in plastic culture dishes at 37° C in medium consisting of 80% Hanks balanced salt solution, 5% horse serum, 5% calf serum, 10% embryo extract, and supplemented with an additional 100 mg% dextrose and 100 U ml^{-1} penicillin-streptomycin mixture. Cultures were incubated in a water-saturated, 3% CO_2 atmosphere at *p*H 7.4. Several cell types were present in the cultures including fibroblasts and neuroglia, but only fully developed motor and association neurones were considered in this study. The neurones were readily identifiable with phase microscopy by their large size, angular cyton with long axon and dendrites, extensively granular cytoplasm, and large vesicular nucleus with prominent nucleolus. Mature 16-d coverslip cultures of fully differentiated neurones were exposed to medium containing ouabain concentrations ranging from 10^{-6} to 10^{-4} M for 3 h, followed by 6 h or 72 h exposure to ouabain-free medium containing 0.05 μCi ml^{-1} of ^{3}H-thymidine. Verification of neurone depolarisation by the ouabain treatment was obtained in separate tests by direct E_m measurements. Mean resting potentials of −60 mV (−40 to −68 mV range) and −12 mV (−8 to −22 mV range) before and after a 2.5 h exposure to 5×10^{-5} M ouabain, respectively, were recorded. Changes in intracellular Na accompanying the depolarisation were determined by electron microprobe analysis of individual neurones. Thymidine incorporation was assayed by autoradiography. The coverslips were rinsed, fixed, alcohol dehydrated, air dried, mounted on microscope slides, and coated with Kodak NTB-3 nuclear emulsion. After an 8 d exposure, the developed slides were stained with a mixture of neurone-specific polychrome and haematoxylin. Neurones were considered labelled only if the emulsion grain density was at least three times background and confined primarily to the area of the nucleus.

Mitotic activity in mature neurones of the CNS ceases completely in the fully differentiated state[6–11]. This finding was also confirmed in our study, for neurones of the chick spinal cord (Fig. 1). Histological sections of cords from 33 h to 10 d *in ovo* showed that neuroblast proliferation was confined to the inner ependymal layer surrounding the neural canal and reached peak activity at 3 d, followed by a steady decline to virtual quiescence by day 10 of incubation. Similar studies of chick dorsal root ganglia have shown that neuroblast divisions begin to decline on the seventh embryonic day and cease by the ninth day[12]. That the neurones used in the present investigation had differentiated beyond the proliferation stage at the time of plating was verified by taking sequential photographs and time-lapse films of numerous marked fields of neurones over several weeks following plating, wherein continued growth and extensive process formation characteristic of fully differentiated neurones were observed, but no mitoses. In rare instances, a binucleated neurone possibly representing an abortive attempt at cytokinesis was observed, as has been reported by others[8,13]. Some cultures remained in a viable state for as long as 20 weeks.

Table 1 ^{3}H-Thymidine Incorporation Induced by Ouabain Depolarisation

Ouabain concentration (M)	^{3}H-Thymidine exposure time (h)	No. of neurones counted	Labelled neurones (%)
10^{-6}	6	372	7.9
5×10^{-6}	6	301	10.4
5×10^{-6}	72	359	9.4
10^{-5}	6	616	11.0
10^{-5}	72	253	14.0
5×10^{-5} *	72	195	15.7
10^{-4}	—	Detached	—
Control †	72	980	1.9

* These cultures exposed to ouabain for 9 h; all other cultures exposed for 3 h.

† Grain density of labelled control cells was much lower than in the labelled test cells which had been exposed to ouabain.

Verification of depolarisation by ouabain treatment was obtained by direct E_m measurements in separate experiments. The percentage of labelled neurones is the average for duplicate slide cultures. Thymidine exposure periods immediately followed ouabain treatment periods. A ^{3}H-thymidine activity level of 0.05 μCi ml^{-1} of culture medium was used in all tests.

The percentages of neurones incorporating ^{3}H-thymidine during the 6 and 72 h periods following treatment with various concentrations of ouabain are shown in Table 1. Significant labelling was induced even by the lowest concentration used (10^{-6} M). In general, the percentage of labelled cells increased with concentration of ouabain and duration of labelling period. The 10^{-4} M ouabain concentration caused neurone swelling (as would be expected upon strong depolarisation[1]) and detachment within 3 h, thus preventing further autoradiographic observation. Although 5×10^{-5} M also produced some swelling, the highest percentage of labelled cells was obtained after a 9 h treatment at this concentration. Only an occasional, lightly labelled neurone was observed in the control cultures exposed to ^{3}H-thymidine for 72 h. The fact that approximately the same percentage of neurones became labelled, whether exposed to tagged thymi-

dine for 6 h or 72 h following the 3 h ouabain treatment (5×10^{-6} and 10^{-5} M), indicates that the stimulatory action begins quite soon after ouabain addition. The grain density of the label was, however, significantly higher in the neurones exposed to ^{3}H-thymidine for 72 h, indicating that synthesis continues beyond the initial 6 h period. The increase in the percentage of labelled cells with increase in concentration and length of exposure to ouabain suggests that an even larger percentage of cells might be induced to label with more optimum rates of depolarisation and treatment durations at the various concentrations. The relative intracellular Na level of neurones treated for 2.5 h with 5×10^{-5} M ouabain was 3.3 times that of untreated control neurones, thus indicating that a pronounced influx of Na accompanies the depolarisation induced by ouabain.

The observed incorporation of ^{3}H-thymidine is considered to reflect synthesis of DNA, rather than of RNA or other metabolites, for several reasons: (1) the labelling began in a relatively short period (6 h) immediately following ouabain treatment, (2) labelling was confined essentially to the area over the cell nucleus, (3) controls exposed to ^{3}H-thymidine for the same periods were practically devoid of label, and (4) all labelling was removed from ouabain-treated cells incubated with DNase before autoradiographic exposure, but was unaffected by treatment with RNase. Although the possibility cannot be ruled out that the observed labelling may reflect thymidine exchange or some form of repair activity rather than true mitogenic replication of DNA, the latter alternative is suggested by the relatively dense grain concentration obtained with the low ^{3}H-thymidine concentration and short autoradiographic exposure period used, compared with the thymidine incorporation previously observed in neuronal DNA repair activity[14]. Also, in continuing studies to maximise the labelling (C. M. Cone and C. D. Cone, unpublished), it was found that decreasing the ouabain concentration to 10^{-6} M produced an increase in the percentage of labelled cells to 58% when the medium osmolarity was simultaneously increased 5% by the addition of NaCl. These conditions eliminated the swelling normally associated with depolarisation, and the results argue against ouabain *per se* being the stimulatory agent. The cells exposed to 10^{-5} M ouabain for 3 h were observed for possible mitotic activity over the 72 h labelling period, but only a few clearly defined mitotic neurones were found within this period; no mitotic neurones were found in the controls. This lack of mitotic activity in the treated cells, however, could be a reflection of incomplete DNA replication or the need for more optimum intracellular ionic conditions for passage from the G_2 stage into actual mitosis than existed in the present tests[15].

The three-fold increase in cellular Na found to accompany the ouabain-induced depolarisation presumably results from an inhibition of the Na-pumping capability of the neuronal membrane, since the capability of ouabain to effectively inhibit the Na-pumping activity of neurones has been demonstrated in numerous studies. By use of continuous-recording Na-specific electrodes, 10^{-4} M ouabain was demonstrated to effectively inhibit the Na-pumping mechanism of snail neurones[16], allowing a rapid and sustained rise in the intracellular Na concentration (doubling of the initial concentration within 8 min). Exposure of squid giant axons to artificial seawater containing 10^{-5} M ouabain caused a marked inhibition of sodium efflux and depolarisation; similar findings were reported in squid nerve cell bodies[17–19]. Our observation that a significant rise in cell Na in fully differentiated neurones is followed by initiation of DNA synthesis is considered of particular interest in view of the recent finding that significant E_m and Na level changes are associated with development of contact inhibition of mitogenesis in monolayer cell cultures[20]. Although further studies are required to determine the precise nature of the DNA synthesis, our results are in accord with the premise that mitogenesis activity may be functionally coupled with E_m-associated cellular ionic levels (particular of Na), and encourages the speculation that mitogenic replication of DNA and possibly mitosis might be induced in mature nerve and muscle by proper stimulatory conditions.

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Fibroblast Chalone and Serum Mitogen (Anti-chalone)

RECENTLY we reported that extracts of diploid human fibroblasts or of the used medium from the cultivation of these cells *in vitro* contained materials weighing between 30,000 and 50,000 daltons which would specifically inhibit the uptake of tritiated thymidine by WI-38 diploid human fibroblasts *in vitro*[1]. This specific and endogenous mitotic inhibitor was termed a fibroblast 'chalone', following the proposals of Bullough and Laurence[2,3].

This paper reports the effects of partially purified fibroblast chalone concentrate on the population generation time of diploid human fibroblasts in culture, and the competitive relationship between this chalone and the purified sialoprotein isolated from mammalian sera which has been shown to be stringently required by diploid human fibroblasts for mitosis *in vitro*[4].

Diploid human fibroblasts derived from either cutaneous biopsy explants or WI-38 (Flow Laboratories, Maryland) were cultivated in minimum essential medium (MEM) containing 10% calf serum and supplemented with glutamine and penicillin–streptomycin in the usual fashion[5]. These cells were counted in a haemocytometer after collecting by trypsinisation and were seeded into Leighton tubes at a concentration of 40,000 cells per 1.5 ml of medium. At 24 h, these fibroblast cultures were rinsed with balanced salt solution and the medium changed, re-introducing fresh MEM supplemented