THE ROLE OF THE SURFACE ELECTRICAL TRANSMEMBRANE POTENTIAL IN NORMAL AND MALIGNANT MITOGENESIS

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INTRODUCTION

The studies reported in the present paper are part of a continuing investigation of the premise that the electrical transmembrane potential (E_m) and associated ionic concentration differences may be functionally involved in the control of mitogenesis in somatic cells. The subject hypothesis initially arose from observations that the (early-G_1) E_m level for a range of actively proliferating cell lines in culture was significantly lower than that of mitotically quiescent (G_1) cells *in vivo*.[1] Although the pronounced differences in E_m level and mitotic activity observed between proliferating cells in culture and such highly polarized, nondividing somatic cells as (*in vivo*) nerve and muscle prompted the initial hypothesis, such E_m data as were available for cells of other (nonexcitable) *in vivo* tissues also supported the possibility that a general correlation might exist between the relative degree of mitotic activity and the E_m level of somatic cells. Also of particular interest in terms of this initial hypothesis were scattered observations that indicated that the E_m level of actively proliferating malignant cells is greatly reduced relative to that of their adjacent normal homologs.[2-4] A formal statement of the subject hypothesis, which proposes an E_m-ionic basis for the modulation of mitogenic activity, has been presented previously with a summary of pertinent initial experimental evidence.[5]

Two primary mechanisms appear to exist by which observed E_m level differences might be involved in effecting control of mitogenic activity. The first category includes direct influences of the E_m-associated intramembrane electric field intensity on such membrane properties and activities as ionic-molecular permeabilities and active transport, activity of membrane-bound enzymes and associated surface reactions and syntheses, control of specific ion availability through sequestering by the membrane, and others, with consequent alterations of intracellular metabolic activities associated with mitogenesis. Much evidence exists, for example, which indicates that the instantaneous (local) E_m level exerts a profound influence on the Na^+ permeability of the membrane in excitable tissues. The second area includes the effects of E_m-associated intracellular ionic concentrations (particularly those of Na and K) on mitogenic activities, which range from direct regulation of (mitogenically essential) RNA transcription[6-8] to regulation (either directly or indirectly) of the activity of mitogenically essential enzymes. Under particular conditions, both mechanisms might be active in varying degrees, and each must be considered in the design of experimental programs to investigate the hypothesis. In our studies, early exploratory experiments[9] on ionic mitogenic effects demonstrated that imposed changes in intracellular ionic concentrations, which simulate those that would (according to the classical Hodgkin-Huxley theory of E_m generation in excitable tissue)

accompany major increases in E_m level, produce complete arrest of DNA synthesis and mitosis in cultured cells (without a significant change in the physical E_m level). Consequently, our experimental studies to date, which include those discussed here, have involved investigation of the correlation of cellular ionic shifts in conjunction with measurements of the E_m level per se under various conditions of mitotic activity.

The present paper discusses the results of measurements of corresponding E_m, ionic concentration, and mitotic activity levels for several diverse cell systems, under conditions of both active proliferation and mitotic quiescence. It also summarizes the results of studies that investigated the effects of imposed depolarizations in stimulating initiation of mitogenic activity in normally nondividing cells. The results indicate that, for all the systems studied to date, a strong and consistent correlation exists between the magnitude of the E_m level and the degree of mitotic activity, and they suggest a direct functional involvement of the associated intracellular ionic environment, particularly of the Na^+ concentration, in mitogenesis control.

Experimental Systems and Investigative Approaches

Preliminary Experiments

If the Hodgkin-Huxley theory of E_m generation in excitable cells is also generally valid for cells of nonexcitable tissues (some evidence does exist to indicate that the essential features, such as active Na^+ pumping, apply[10-12]), substantial changes in E_m level would be expected to be accompanied by corresponding shifts in the intracellular Na and K concentrations, under new steady-state conditions. Consequently, precepts of the classical Hodgkin-Huxley theory were utilized as the basis for design of preliminary experiments to ascertain if the expected intracellular ionic conditions that accompany various E_m levels would affect mitogenic activity. Naturally synchronized monolayer populations of Chinese hamster ovary (CHO) cells were immersed in culture media that had Na^+ and K^+ concentration levels which simulated those of muscle cytoplasm at high potential levels (-50 to -90 mV), with the expectation that because the ion-pumping activity of the actively proliferating CHO cells was already quite low, as evidenced by the low E_m value of -10 mV, they would assume essentially the same Na^+/K^+ concentrations intracellularly as existed in the external medium. The results[9] demonstrated that imposed ionic conditions that simulate those which would accompany an E_m level of ~ -65 mV fully, but reversibly, arrested mitotic activity in the G_1 phase, as usually occurs in normal mitotic arrest *in vivo*. Since the very small physical E_m level of the untreated cells (-10 mV) changed only slightly in the "high-potential medium" used (-6 mV), it appeared that the observed mitogenic effect was associated more directly with the change in ionic conditions than with a change in the physical E_m level per se. Because previous experiments that investigated the effects of various ions on the progress of the cell cycle had revealed that high Na^+ levels were specifically capable of producing a significant shortening of the interphase period and increasing the degree of mitotic synchrony in mitotically active populations,[1] it was concluded that the variation of the Na^+ concentration level might be a factor of primary importance in the observed arrest of mitogenesis in media that have very low Na^+ concentrations. Because of these encouraging preliminary results, the further investigations reported here have been performed with several diverse cell systems under comparative mitotic conditions in an effort to determine if a degree of generality exists for a correlation between E_m-ionic levels and mitogenic activity.

Systems and Approaches

Essentially simultaneous measurements of the E_m level, Na and K concentrations, and the mitotic index of various systems are required for comparison of cells of a given system under both the mitotically active and mitotically quiescent conditions, to establish if a correlation between the E_m and ionic levels, and mitogenic activity exists. Our experimental investigations have thus been based on examination of cell systems of two general types: systems in which initially mitotically active cells enter, as a result of either natural or artificial influences, a state of mitotic arrest; and systems in which initially mitotically quiescent cells are induced by either natural or artificial means to resume mitotic activity. Examples of the first type are: proliferating monolayer cultures (and similar clonal colonies), which eventually develop contact inhibition of mitosis as culture saturation densities are attained; development of embryonic organ and tissue systems (e.g., nerve, muscle, liver) to the mature stage; and wound healing. Examples of the second type are: mitotically contact-inhibited saturation cultures that are stimulated to divide by physical or chemical means (e.g., "wounding" of saturated monolayers or serum, trypsin, and other treatments, respectively); mitotic activation of *in vivo* cell systems after physical wounding or hormone (e.g., estrogen) treatment; and sustained proliferation induced by malignant transformation of normal cells. What might be classed as a third type of system, also under investigation, consists of cells that are normally mitotically active, or mitotically quiescent, which are induced to enter the mitotically quiescent, or mitotically active state, respectively, by imposition specifically of E_m or ionic levels that, in terms of the hypothesis, would be expected to be mitogenically effective. Examples are stimulation of mitogenesis in mature CNS neurons and in muscle cells by sustained depolarization and blockage of mitogenesis by imposition of ionic conditions that simulate high E_m levels (as in the "preliminary experiments" already described).

The specific studies for which results are summarized in this paper have utilized the following *in vitro* systems: development of contact inhibition of mitosis in monolayer cultures and in clonal colonies of various cell lines, release of saturated cultures from contact inhibition by monolayer wounding and by addition of fresh serum, stimulation of mitogenesis in mature CNS neurons by sustained depolarization, and stimulation of increased mitotic activity in log-phase monolayer cultures by trypsin.

For the studies reported here, E_m measurements were made by direct impalement with glass microelectrodes; the techniques and electrometer system used have been previously described.[1,8] Mitotic activity, expressed in terms of the mitotic index (fraction of population that divides per unit time), was generally determined by direct observation from time-lapse cinematography, with additional verification where appropriate by counts of metaphase cells after a 4-hr colchicine treatment. DNA synthesis determinations were performed by autoradiographic analysis after pulsed incubation with [^{3}H]thymidine. Determinations of cellular Na and K contents were made by atomic absorption spectroscopy of pooled cell populations. In some cases, however, the relative Na contents of mitotically active and mitotically quiescent cells were determined from electron probe microanalysis of single cells. Although somewhat impractical for analysis of large numbers of cells, the microanalyzer is exceptionally valuable for direct comparison of cytoplasmic ionic contents of individual cells in localized areas of intact (freeze-dried) monolayers and colonies. Other pertinent aspects of test procedures are discussed under the specific experiments in RESULTS.

Results

Development of Contact Inhibition of Mitosis In Vitro

Monolayer Development

Cell lines that develop contact inhibition of mitosis at saturation densities provide excellent *in vitro* experimental systems for measuring E_m and ionic levels under mitotically active and quiescent conditions. In this study, essentially simultaneous measurements of E_m, cellular Na and K, and mitotic activity were performed with methods previously described,[8] for both the CHO and 3T3 cell lines as the cultures progressed through log-phase proliferation to the contact-inhibited state at saturation. CHO monolayer cultures maintained on plastic windows of perfusion chambers were monitored by time-lapse cinematography to determine the simultaneous local cell density and mitotic activity; E_m measurements were performed at corresponding times on cultures that were replicates of those filmed. Monolayer cultures of 3T3 cells were maintained in plastic petri dishes and (average) cell densities per plate obtained by electronic counting of trypsinized plates; E_m measurements were made from replicate dish cultures.

The results for the CHO line (FIGURE 1) reveal that at the lower density levels (i.e., up to 26 hr) the cells maintain essentially log-phase growth and the low E_m level characteristic of actively proliferating cultured cells (−11 mV). As the cell density increases, however, and substantial direct cell-to-cell surface contact begins to de-

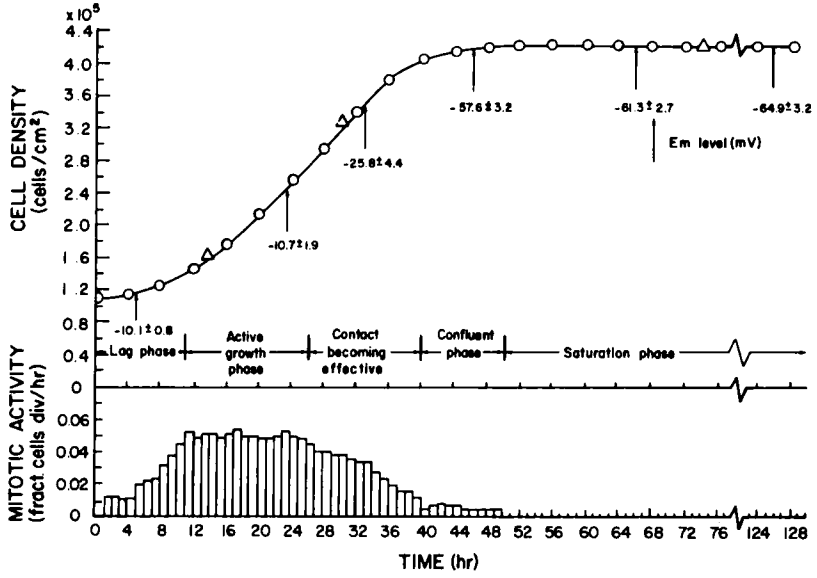

FIGURE 1. Variation of cell density, E_m, and mitotic index levels with time in developing CHO monolayer cultures. Density and mitotic results are pooled data from three time-lapse films of selected areas of monolayer cultures; groups of 1600–5800 cells analyzed per data point. ○, counts from time-lapse films; △, electronic counts of whole chambers. The E_m value at each density condition is the mean (±SD) of ≈ 150 cell measurements.

velop (as verified by electron microscopy), mitotic activity begins to decrease steadily, with a corresponding rise in the (average) E_m level. The E_m level of individual cells at this stage appears to be directly related to the extent of their surface contact with other cells, with loosely contacting cells on the edge of developing confluent areas having substantially lower E_m levels than cells in the interior of such areas; the local mitotic activity directly mirrors the distribution of E_m levels in the culture. As the cell density continues to rise to the full saturation value, mitotic activity decreases essentially to zero as the E_m level attains a value of -61 mV, a level which parallels that of many mitotically quiescent *in vivo* cell types.[13] The E_m level continues to rise slightly with time to a final value of -65 mV; this additional rise appears to be associated with consolidation of the confluent CHO cells in a matrix of extracellular material.

The results for the 3T3 line (FIGURE 2) are essentially the same as for the CHO line (FIGURE 1), except that the E_m and mitotic activity changes associated with the cell density increase become apparent at much lower density levels (i.e., at 120 hr). (As has been pointed out previously, however, this presumed "high mitotic sensitivity" of the 3T3 line to surface contact may not really exist; in view of the larger volume and surface area of these cells relative to those of the CHO line, both lines may actually possess about the same amount of contacting surface area per unit of culturing surface area.[8])

Determinations of cellular Na and K for both cell lines in the log and saturation phases (TABLE 1) reveal that a significant decrease in cellular Na accompanies the E_m rise at saturation; only slight changes occur in cellular K. Because only *total* Na and K were measured, these concentrations cannot be directly related to the measured E_m in terms of the electrochemical potential difference. The significant Na decrease that accompanies the E_m rise is, however, qualitatively in agreement with the Hodgkin-Huxley theory.

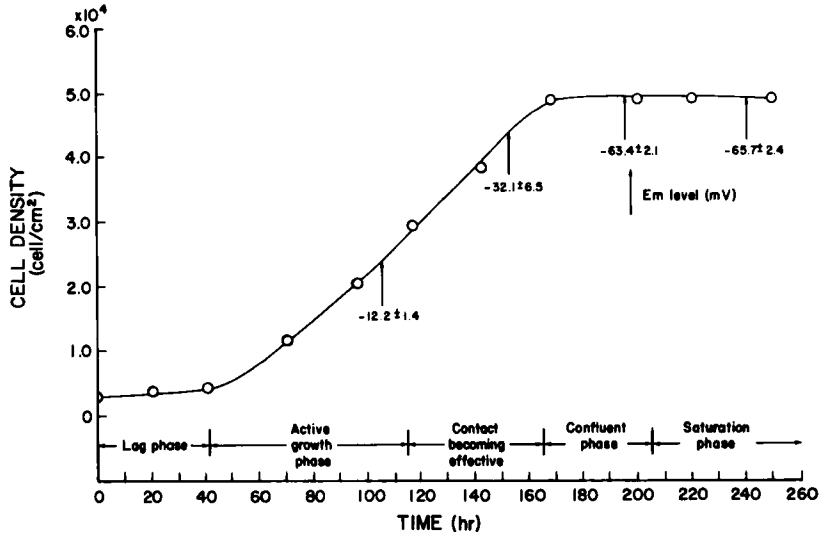

FIGURE 2. Variation of cell density and E_m levels with time in developing 3T3 monolayers. Each density point is the mean of counts of three replicate dishes; each value is the mean ($\pm$SD) of ~150 cell measurements.

TABLE 1

NA AND K CONCENTRATIONS IN LOG PHASE AND SATURATED CHO AND 3T3 MONOLAYERS*

Ion	CHO		3T3	
	Log	Saturated	Log	Saturated
Na^+	15.3 ± 1.8	7.9 ● 2.1	17.6 ± 1.5	8.6 ± 0.8
K^+	186.1 ± 5.3	185.9 ± 6.2	204.5 ± 3.6	197.0 ± 4.8

*Concentrations are expressed as μmol of ion/ml of mean total cell volume ± SD. Each value is the mean of six separate determinations; separate mean cell volume determinations were made for each test set, which correspond to each value.

These results demonstrate that a significant increase in E_m level is associated with development of mitotic arrest in saturated cultures; thus, it is concluded that the mitotic arrest induced by contact inhibition phenomena *in vitro* follows the hypothesized E_m-mitotic activity correlation. Moreover, the substantial decrease in Na that accompanies this E_m increase suggests that a primary effect of cell surface contact may be the alteration of ionic transport activity by changes in either specific membrane permeabilities or ion-pumping activities, or both.[8] The fact that two different cell lines with supposedly different "contact sensitivities" appear to behave in an almost identical manner in regard to E_m and ionic concentration changes at saturation densities suggests some generality for the involvement of the E_m-mitotic activity correlation in contact inhibition of mitosis in cell systems.

Colony Development

Local E_m, density, and mitotic activity measurements have also been made as functions of radial position in clonal colonies of CHO and 3T3 cells.[8] The results are practically identical with those found in developing monolayers, despite the differences in the manner in which saturation density is attained in the two culture forms. Mitotic activity ceases in the colony centers when the density there reaches the normal monolayer saturation value; the accompanying E_m level that exists at this time is the same as that found in saturated monolayers. Mitosis continues actively in a peripheral band of loosely contacting cells, where the E_m level is the same as that of free log-phase cells in monolayer cultures.

Release from Contact Inhibition of Mitosis In Vitro

Physical Release

This series of experiments was designed to investigate the reverse of the conditions of monolayer development by inducing proliferation in contact-inhibited cells by "wounding" a saturated culture (i.e., production of a cell-free gap in the monolayer). In this procedure, reactivation of mitosis is produced entirely as a result of physical breakage of surface contact per se. Gaps of approximately 1000 μm width were produced with a plastic microspatula in saturated monolayers of CHO cells maintained on plastic windows of perfusion chambers, and the subsequent activity of the cells at the gap margin was followed in time-lapse cinematography. By this method, continuous recordings of local cell density and mitotic activity relative to the edge of the cell sheet were obtained. E_m measurements were made at

selected times in 100 μm wide strips of cells at increasing distances from the leading edge of the cell sheet in cultures that were replicates of those filmed.

After gap production, the cell population immediately adjacent to the gap is seen to move toward the gap as a continuous layer, similar to the expansion of a previously compressed rubber sheet, with a clearly discernible decrease in local cell density as the expansion occurs. Ultimately, some cells on the leading edge detach from the sheet and move into the free area of the gap. The E_m level of many of the cells on the immediate leading edge of the sheet (the first and second rows of cells) has decreased to approximately −12 mV within 2 hr after wounding, and cells immediately behind these edge cells also have decreased E_m levels (TABLE 2); the remaining peripheral cells at this time retain the same saturation E_m level as the original undisturbed monolayer. Measurements at increasing distances behind the leading edge of the sheet at 8, 12, 16, and 24 hr show a definite correlation between the local cell density and E_m level; the conditions are very much similar to those in the peripheral region of developing colonies of CHO cells, with the (average) E_m level decreasing continuously from that of the undisturbed sheet to that of the free cells at the leading edge. The relationship between E_m level and cell density is indicated in FIGURE 3.

The first mitoses begin at approximately 14 hr and occur in cells that had been on the immediate edge of the initial wound (the first cells to lose contact and depolarize); shortly thereafter, mitoses begin in cells within the still confluent, but low-density, areas of the sheet edge. Correlation of mitotic activity with the local cell density and E_m level in the wound-edge cell system is somewhat complicated by the fact that the cell sheet is continuously expanding and the local density continuously changing; thus, direct association of a given mitosis with the precise density and E_m conditions that existed *at the time of, and presumably stimulated, its resumption of mitogenesis* is difficult. The procedure that has been used to establish this correlation is based on the premise that once a previously G_1-arrested cell has been stimulated to resume mitogenesis by the occurrence of loss of effective contact and depolarization at a given time, a period slightly less than the normal cycle period will be required before mitosis is observed. Consequently, in the present experiments, each mitotic cell observed in time-lapse cinematography was traced back for a period of 13 hr (0.95 times the normal log-phase cycle period of the CHO cells used) and the local cell density and E_m at that time determined. The results (FIGURE 3) indicate that the major fraction of the cells that divide during the observation period

TABLE 2

DEPOLARIZATION AND CELL DENSITY CHANGES AT GAP EDGE IN MONOLAYER WOUND

Time After Wounding (hr)	Cell Density (cells/cm^2)	E_m (mV ± SD)	Number of Cells Impaled
2	free*	−11.4 ± 1.4	24
	3.4×10^5†	−22.0 ± 3.1	39
4	free*	−11.6 ± 1.1	32
	3.1×10^5†	−23.9 ± 2.8	30
8	free*	−12.8 ± 1.1	23
	3.6×10^5†	−29.6 ± 3.5	25

*Free cells at edge.

†Cells in 100-μm strip behind edge.

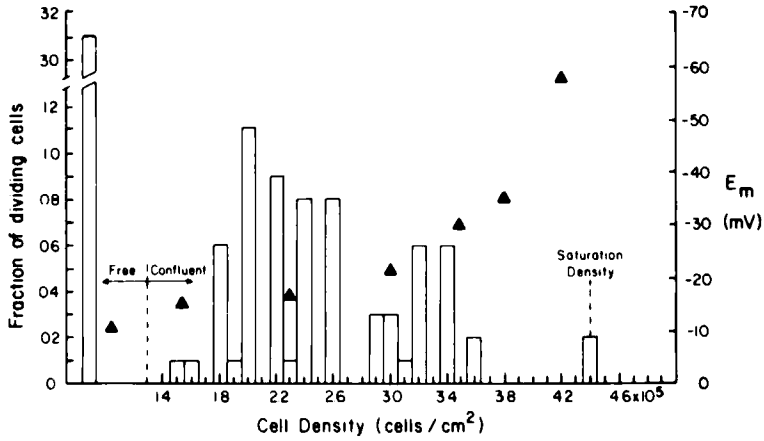

FIGURE 3. Correlation of mitotic activity in edge cells of a monolayer wound with the E_m and cell density conditions that exist in their immediate environment approximately one cycle period (13 hr) prior to division.

were located in low-density, low-E_m areas of the cell sheet one cycle period prior to division. Most of the dividing cells were still in confluent areas at the time of activation but were in regions of low cell density. These results again indicate a direct relationship between cell density, the E_m level, and the degree of mitotic activity and demonstrate that arrested mitogenic activity can be reactivated simply by disrupting or sufficiently lowering the extent of physical cell-to-cell surface contact.

Chemical Release

Addition of medium that contains fresh serum to saturated monolayer cultures results in a stimulation of mitosis in a small percentage (6–15%) of the cells approximately one cycle period later.[14] A recent investigation in which continuous E_m recordings were made during addition of fresh serum to saturated monolayer cultures[15] revealed that a pronounced depolarization of the cells occurs within a few minutes after serum addition and is maintained for up to 3 hr, during which a gradual increase in E_m back to the initial level occurs. A fraction of the cell population ultimately commences DNA synthesis. These observations again imply that a direct correlation exists between cellular depolarization (in this case, chemically induced) and mitogenic activation. The question arises, however, as to why only a fraction of the cells ultimately divide when depolarization is apparently initially induced in practically all of the cells. In an effort to obtain some insight into this question, experiments were performed in which the E_m level and relative cytoplasmic Na content of 3T3 cells were measured at 0.5 and 4 hr after addition of fresh serum. The relative Na contents were determined for individual cells by electron microprobe analysis of washed, freeze-dried monolayers. Na measurements were made on individual cells rather than on pooled cultures, because any change that might occur in the Na content of the small fraction of cells (8.5%) stimulated to divide might be masked by the much larger nondividing portion of the population.

The results of these measurements are shown in TABLE 3. At 0.5 hr after serum addition, only 8% of the measured cells have E_m levels larger than -20 mV, and only

TABLE 3

E_m AND Na LEVELS IN SERUM-TREATED CELLS

Time (hr)	$E_m > -20$ mV (%)	[Na] > 1.25 × Control (%)
0.5	8.0	1.0
4.0	90.9	8.7*

*The Na concentration in these cells was > 1.8 times larger than that in the control cells.

1% have Na levels larger than 1.25 times that of the control cells (i.e., untreated, saturated monolayer replicates of the test cultures). At 4 hr, 90.9% of the cells have E_m levels larger than −20 mV, the average value approaching the saturation E_m level, and 8.7% have Na levels larger than 1.8 times that of the control cells. It is obviously not possible to determine directly if the cells that have low E_m values at 4 hr are the same cells that contain high Na levels at this time or if either of these groups represent the actual stimulated cells that will eventually divide. However, the similarities of the percentages of cells in these groups with the total percentage of cells that ultimately divide after serum treatment (8.5%) are suggestive of such a correspondence. The finding that a small fraction of cells, equivalent in value to the fraction that ultimately divides, remains in the depolarized state while most of the population has fully repolarized 4 hr after serum treatment suggests a possible explanation as to why most of the population does not divide. Presumably, the transient depolarizing action of fresh serum is too short in duration to initiate sustained mitogenesis in the majority of the cells but is sufficiently prolonged in a small fraction of the cells to permit full activation to occur. In view of the foregoing results that suggest an involvement of Na in mitogenesis stimulation, this requirement for a prolonged depolarization might be interpreted as the time required for the cellular Na concentration to increase to a mitogenically active level and initiate sustained mitogenic activity.

Stimulation of DNA Synthesis in Mature CNS Neurons by Sustained Depolarization

If the E_m and associated ionic levels are in fact involved in mitogenic control as hypothesized, presumably it should be possible to initiate mitogenic activity with ultimate mitosis in such highly polarized, normally nondividing cells as CNS neurons and muscle by sustained depolarization, *provided* the depolarization can be accomplished and the lowered E_m level maintained in such a manner that the essential metabolism of the cell is not so adversely affected that normal mitogenic activity is precluded. The possibility has been investigated for the case of mature, fully differentiated CNS nuerons of the chick spinal cord by depolarization with the cardiac glycoside ouabain.[16] Mature 16-day-old cultures of fully differentiated neurons obtained from the spinal cords of 7–10-day chick embryos, cultured after the method of Fischbach,[17] were exposed to culture medium that contained ouabain concentrations ranging from 10^{-6} to 10^{-4} M for 3 hr, followed by a 6- or 72-hr exposure to ouabain-free medium that contained 0.05 μCi/ml of [^{3}H]thymidine, with subsequent autoradiographic analysis. Untreated control neurons had a mean E_m level of −60 mV, with values ranging from −40 to −68 mV, and were devoid of both DNA synthesis activity and mitoses; the ouabain treatment resulted in an almost complete depolarization (to −4 mV) within 0.5 hr for the higher concentrations.

The results of the initial experiments are given in TABLE 4. Significant labeling is induced even by the lowest ouabain concentration used (10^{-6} M). The percentage of labeled cells generally increases with ouabain concentration and length of the labeling period. Although cellular swelling accompanied the rapid depolarization induced by the higher ouabain concentrations ($>10^{-4}$ M), the highest percentage of labeled cells was obtained after a 9-hr treatment with 5×10^{-5} M ouabain. Verification that the observed labeling represents [^{3}H]thymidine incorporation specifically into DNA was obtained by demonstrating that incubation of ouabain-treated cells with DNase prior to autoradiographic exposure removed all label from the cells; the label was unaffected by incubation with RNase. Only a rare, faintly labeled neuron was observed in control cultures exposed to [^{3}H]thymidine for 72 hr. These initial results clearly demonstrated that the conditions that accompany the sustained depolarization induced by ouabain are capable of stimulating DNA synthesis in mature neurons, and the increase in the percentage of labeled cells with increase in ouabain concentration and length of exposure suggested that an even larger percentage of cells might be induced to label with more optimum rates of depolarization and treatment durations at the various concentrations to prevent swelling of the cells. The relative cytoplasmic Na levels of control and ouabain-depolarized neurons were determined for individual cells with electron microprobe analysis. The Na level of the neurons treated for 2.5 hr with 5×10^{-5} M ouabain was 3.3 times that of the untreated controls, which thus indicates that a pronounced influx of Na accompanies the depolarization induced by ouabain.

In an attempt to alleviate the substantial swelling of the neurons caused by the ouabain-induced depolarization, further studies that endeavored to optimize the treatment duration and osmotic balance have been performed for a range of ouabain concentrations. The best results to date[18] have been obtained with the use of the lower ouabain concentrations coupled with a slight increase in medium osmolarity obtained by addition of NaCl. With a ouabain concentration of 10^{-6} M applied for 3 hr and a 5% increase in medium osmolarity obtained by NaCl addition, 58% of the cells have been induced to label heavily with a 7-hr [^{3}H]thymidine labeling period and a 7-day autoradiographic exposure period. Under these conditions, neuron swelling was eliminated. A slight increase in the percentage of neurons that synthesized DNA (8% labeled cells) above the control level was induced by the in-

TABLE 4

[^{3}H]THYMIDINE INCORPORATION INDUCED BY OUABAIN DEPOLARIZATION*

Ouabain Concentration (M)	[^{3}H]Thymidine Exposure Time (hr)	Total Neurons Counted	Labeled Neurons (%)
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

*Depolarization by ouabain treatment was verified by direct E_m measurements. The percentage of labeled neurons is the average for duplicate slide cultures. An [^{3}H]thymidine activity level of 0.05 μCi/ml of culture medium was used in all tests.

clusion of additional NaCl (5% osmolarity increase) alone in the medium (without ouabain addition). Swelling and cell death occurred, however, in attempts to further increase the labeling by use of higher NaCl osmolarities, which thus indicates that the cells are quite sensitive to excess Na levels in the bathing medium. The density of labeling induced in the cells in these more recent experiments is strongly suggestive of mitogenic replication of DNA rather than simple replacement or repair synthesis.

These results appear significant from two standpoints. First, the substantial labeling obtained by sustained depolarization agrees fully with the predictions of the basic hypothesis and thus appears to offer strong support for the existence of a functional relationship between the E_m-ionic level and mitogenic activity. Second, if complete mitogenic replication of DNA can be effected by sustained depolarization in neurons (and perhaps in muscle as well), the possibility of inducing complete mitosis in such cell forms arises, with the attendant possibilities for stimulation of regenerative growth in these important nondividing tissues.

Potential Involvement of the Na Level in Mitogenesis Control

It appears significant that substantial changes in the cellular Na level have been found to accompany the E_m-mitotic activity level changes observed in most of our experiments to date. The results suggest that the intracellular Na level may be a factor of basic importance in the regulation of mitogenesis activity in somatic cells and may have a more direct influence on mitogenic metabolic events than the physical E_m per se. The occurrence of Na concentration changes in association with E_m changes is of course to be expected in terms of the Hodgkin-Huxley theory for excitable cells, with its central emphasis on active Na transport. Although insufficient quantitative evidence is presently available for concluding the general applicability of the Hodgkin-Huxley system of E_m-ionic relationships to all nonexcitable tissues, such results as do exist[10–12] tend to confirm the operation of a Na-pumping system in several nonexcitable cell types. Conversely, evidence has been advanced for the existence of pumping systems for a variety of other ions, so that measured E_m alterations in a given cell type cannot always be considered unequivocally as indicative of corresponding alterations in the intracellular Na (or K) levels, and vice versa. Two of our experiments have revealed significant changes in the intracellular Na level, with pronounced mitogenic consequences, but without appreciable change in the E_m level. For example, in the preliminary "high-potential medium" experiments already cited, in which complete but reversible blockage of DNA synthesis and mitosis were obtained, the intracellular Na level was significantly decreased, but only a small change in the E_m level was observed. Similarly, in experiments that investigated the phenomenon of trypsin acceleration of log-phase cellular proliferation,[19] it was found that although addition of trypsin to the culture medium produced a three-fold increase in intracellular Na and a pronounced increase in the proliferation rate in CHO cells, the low basic (G_1) E_m level of the cells (−9 mV) remained unaffected by trypsin addition.[20]

The present results, which suggest that Na level changes may be associated with, or directly involved, in mitogenesis control, are of particular interest in view of the findings of Congote and colleagues[7] that the extranuclear Na concentration exerts a pronounced influence on nuclear RNA polymerase activity; the K concentration was found to be without effect. Similarly, Kroeger[6] has found that the Na/K concentration ratio directly controls chromosome puff formation and synthetic activity and that the entire sequence of chromosome-puffing patterns which occurs in

normal morphogenetic development can be precisely reproduced by imposed stepwise changes in the environmental Na/K ratio. He has also found[21,22] that Na (specifically) is capable of reverting the pattern of gene activities of mature, differentiated cells to those characteristic of the more immature stages of cell maturation. These specific regulatory capabilities of the Na concentration, which act at the very basic genetic level, suggest an attractive possible mechanism by which mitogenesis control might be effected in somatic cells and provide some insight as to how observed E_m levels may be functionally coupled with mitogenic metabolism. Under normal (i.e., mitotically quiescent) conditions *in vivo,* the cell surface maintains functional contact with adjacent cells, and its Na permeability and Na extrusion rate are such that a low Na level (and high E_m level) is maintained. This low Na level is then presumed to prevent synthesis of one or more mitogenically essential species of RNA, and mitogenesis consequently remains in an arrested state. Ultimately, when some stimulating influence in the cellular environment, by whatever mechanism, acts to alter either the Na permeability or the Na extrusion rate or both, such that the Na level rises to a sufficiently high level (i.e., the depolarized state) to activate synthesis of the required RNA species, mitogenic activity resumes, with subsequent mitosis. In this scheme, the "mitotic block"[23] occurs at the fundamental RNA synthesis state and is applied very early in the G_1 phase of the cycle. Presumably, the mitogenic stimulatory influences would have to maintain the cell in a sufficiently depolarized state long enough for adequate RNA synthesis to occur in order to initiate sustained mitogenesis. In this control scheme, the cell surface and its ionic transport hierarchy obviously occupy a position of foremost importance. The essential features of the control mechanism proposed here are outlined schematically in FIGURE 4.

SUSTAINED DEPOLARIZATION AND MALIGNANT PROLIFERATION

Another example from the class of cell systems in which mitotically quiescent cells are "stimulated" into mitotic activity is malignant transformation of normal cells *in vivo,* which constitutes a system of particular significance in the present investigation of E_m-ionic involvement in mitogenesis control. Although only quite

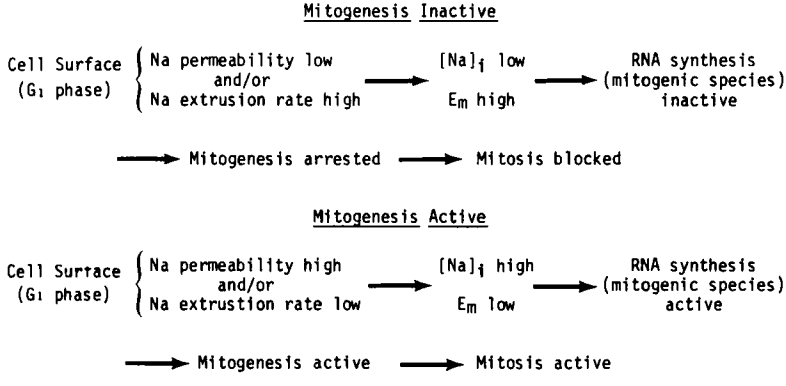

FIGURE 4. Schematic diagram of hypothetical chain of conditions involved in mitogenesis control in somatic cells.

limited information presently exists on E_m-ionic levels and the simultaneous mitotic activity of malignant cells, those measurements that have been made indicate that a pronounced depolarization exists in malignant cells relative to their normal homologs.[2–4,12] One of the more complete studies in this regard[2] demonstrated that a gross depolarization and elevation in intracellular Na occurred in an actively proliferating sarcoma, compared to the adjacent (mitotically quiescent) normal tissue. These observations of a pronounced depolarization coupled with sustained mitotic activity in malignantly transformed cells are in direct agreement with the present findings for normal cell systems and suggest that malignant proliferation may in fact be merely a particular exemplification of the general hypothesis, wherein some basic aberration blocks or negates the effective functioning of the ionic regulatory system and thus results in a sustained depolarization of the cell with associated inability to lower the Na concentration to nonmitogenic levels.

Cellular aberrations that would presumably block effective ionic partitioning (i.e., maintenance of low Na and high E_m levels, in terms of the present hypothesis) and thus lead to sustained proliferation could have many forms. In principle, any sustained aberration that would decrease the membrane permeability to Na or block or reduce the effective operation of the Na pump, would result in continuous (uncontrolled) proliferation. Among the factors that could conceivably affect both membrane permeability and the function of the Na pump are specific molecular structural aberrations in either membrane components or enzyme moieties associated with maintenance of membrane permeability and ion transport activity; such molecular aberrations could in turn be caused by direct genetic mutations produced by chemical or physical agents, viral cytogenetic interference, or direct alterations of molecular structure and/or function by carcinogenic agents. Also, because the Na pump additionally requires energy for its effective operation, any factor that would reduce the energy levels required for active transport would presumably be effective in stimulating mitogenesis, provided, of course, that the reduction in overall available cellular energy was not so great as to preclude the progress of mitogenic metabolism itself. Warburg and associates[24] have placed great emphasis on the importance of the apparent energy deficit in cancer cells; the present hypothesis offers a tentative explanation as to how such an energy deficit might lead to sustained proliferation in malignant cells.

In conjunction with the characteristic of sustained, uncontrolled proliferation, the property of invasiveness (which ultimately gives rise to metastases) defines the malignant state. In seeking the more probable form(s) of aberration that underlie sustained malignant proliferation from among the great number of possibilities that exist, a consideration of invasiveness appears to offer some valuable leads. Much evidence exists to demonstrate that malignant invasiveness is intimately associated with functional aberrations of the cell surface in regard to contact and adhesion phenomena. Because the cell surface is also intimately involved in ion transport, and dysfunction of the ion transport hierarchy has been suggested as a key factor that underlies the uncontrolled proliferation of malignancy, it appears most probable that the two major pathological features of malignancy are not only functionally coupled but arise from the same fundamental aberrancy, viz., alteration of the molecular architecture of the cell surface, particularly in regard to the functionality of those moieties associated with cell surface contact and adhesion. The significance of surface contact per se in modulating E_m-ionic levels and mitogenic activity has been clearly demonstrated by the experiments on contact inhibition of mitosis in normal cell systems, and these and other results that involved loss of or substantial reduction in contact inhibition of mitosis in malignant cells strongly suggest that

loss of contact and adhesive specificity by the malignant cell surface may constitute a primary basis not only of invasiveness but also of sustained proliferation. This implication that such changes in the cell surface constitute the key functional aberration that underlies malignant behavior is strengthened by results from numerous recent biochemical studies, which have demonstrated that significant structural changes in normal cell surface polymers (primarily in the carbohydrate moieties of glycoproteins and glycolipids) are produced as a result of malignant transformation by a variety of oncogenic viruses.[25,26] The importance of carbohydrate polymer structure in cellular aggregation and association has been pointed out previously.[27] The biochemical studies cited above additionally reveal the significant fact that the characteristic molecular aberrations seen in the surface polymers are found not only in the surface but in *all* membrane systems of the transformed cells. This finding appears to be of interest in regard to two basic questions of carcinogenesis, which will be briefly mentioned here. First, it suggests a possible explanation of the underlying basis of Warburg and colleagues' observations on the low level of oxidative metabolism in malignant cells, despite the fact that all the requisite enzyme systems are still present in such cells.[28] Presumably, the same altered polymers that result in surface adhesion loss in malignant cells also become incorporated into mitochondrial membranes, where they supposedly interfere with proper enzyme attachment or function, with consequent impairment of effective oxidative metabolism. The resulting decrease in the available energy level of the cell would then be expected to promote sustained proliferation by decreasing the activity of the Na pump. Such proliferation-inducing effects that stem from the supposed mitochondrial membrane defectiveness would augment those associated with adhesion or contact-loss phenomena at the surface area per se, which produce a strong tendency for continuous proliferation.

The second question concerns the mechanism by which the malignant state might conceivably be maintained in a cell without the occurrence of a fundamental genetic mutation or the permanent incorporation of oncogenic viral genes into a host cell's genome. In view of the finding that aberrant polymers associated with malignant transformation are produced and incorporated into all membrane systems of the cell, the following hypothetical mechanism is suggested as a means whereby the malignant properties of the cell might be sustained after complete removal of the initial transforming influence (i.e., chemical or physical carcinogenic agents or viruses). This mechanism rests on the fact that if a particular polymer (or component thereof) requires specifically for its continued synthesis a membrane or template surface that contains in its structure the particular polymer itself as a required functional element for the synthesis, then once that polymer has become completely omitted from or replaced in the template surface by an aberrant (nonfunctional) homolog, synthesis of the original polymer cannot occur, even though all enzymes required for its synthesis may be present. Consequently, if a cell is exposed to some agent that, for example, blocks the action of an enzyme that is required for some stage of the normal polymer synthesis (e.g., a specific transferase) and an aberrant polymer is produced, which then replaces the original polymer in the template in the course of new membrane formation or membrane-turnover replacement activity, once the original polymer has been completely replaced by the aberrant form it cannot thereafter be synthesized by the cell, even though the original blocking agent subsequently is entirely removed from the cell. If the aberrant polymer is capable of acting as a template for its own synthesis, its production will be stably maintained. Obviously, the hypothetical blocking agent cited above could as readily be of viral origin and the aberrant substitution polymer could be partly or

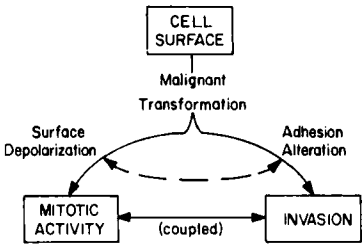

FIGURE 5. Schematic diagram that illustrates the proposed functional coupling of the invasiveness and uncontrolled proliferation of malignancy and that indicates their common source as aberrations in the molecular architecture of the cell surface.

entirely a product of viral gene action; consequently, in such a system, the malignant properties originally induced by viral action could be maintained indefinitely after complete removal of all cytogenetic influences of the virus. Presumably, because there are no basic genetic changes produced in the cell in this scheme, the malignant state could be reversed to the normal conditions by inducing substitution of the aberrant polymer with its normal homolog. Although no direct experimental evidence for the operation of such self-synthesis template systems in cells presently exists, the possibility of this or some similar mechanism is at least suggested by indications that significant polymer abnormalities that result from malignant transformation are found in all membrane systems of the cell and that these abnormalities apparently continue undiminished after removal of the original disturbing influence. In view of the known involvement of carbohydrate aberrations in malignant transformation, it appears that alterations in the normal pathways of glycopolymer synthesis might be a prime area for investigation in regard to the basic mechanism of malignant transformation, with particular attention given to the membrane system of the Golgi apparatus as a possible center for the hypothetical template surface aberrations.

A summary of the basic percepts outlined above, which views the factors involved in malignant proliferation as a particular case of the more general hypothesis of E_m-ionic control of mitogenesis, is given schematically in FIGURE 5.

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